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Deutschland



Isolation and Characterization of Multipotent Postnatal Stem Cells from the Alveolar Bone and Gingival Cervical Margin

INAUGURAL-DISSERTATION

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gez.

Our own well being rests in the smiles and happiness of some other people. It is for the sake of these people only that one should exert himself and try to give in return as much as one has received...

to Shadr

and little Aly

1. Introduction and review of literature	6
1.1 The periodontium	6
1.2 Periodontitis and periodontal regeneration	6
1.3 Embryonic and adult stem cells.....	11
1.4 Types of adult stem cells currently investigated in the dental field	15
1.4.1 Bone marrow stromal stem cells (BMSSCs)	15
1.4.2 Adipose-derived stromal cells (ADSCs)	16
1.4.3 Adult dental pulp stem cells (DPSCs).....	17
1.4.4 Dental follicle stem cells (DFSCs)	18
1.4.5 Stem cells from human exfoliated deciduous teeth (SHEDs).....	19
1.4.6 Periodontal ligament stem cells (PDLSCs).....	20
1.5 Identification criteria for PDLSCs.....	21
1.6 Criteria for the identification of MSCs.....	22
1.7 Aim of the thesis.....	23
2. Material and Methods.....	24
2.1 Sample size and characteristics	24
2.2 Cell isolation and cultivation protocols.....	24
2.2.1 1 st Protocol	24
2.2.2 2 nd Protocol	25
2.3 Cell Passage	25
2.4 Magnetic activated cell sorting (MACS).....	26
2.4.1 MACS Method.....	26
2.5 Flow cytometric analysis	27
2.6 Cryopreservation of the cells	28
2.7 Colony-forming unit (CFU) assay	28
2.8 Osteogenic, adipogenic and chondrogenic differentiation	28
2.8.1 Osteogenic differentiation	29
2.8.2 Adipogenic differentiation	30
2.8.3 Chondrogenic differentiation	30
2.9 Gene expression profile	30
2.9.1. RNA extraction and purification	30
2.9.2 Reverse transcription.....	31
2.9.3. Real-time Polymerase Chain Reaction (real time-PCR)	31
3. Results.....	34
3.1 Phase contrast inverted microscopy.....	34
3.2 Flow cytometric analysis	35
3.3 Colony forming unit (CFU) assay	37
3.4 Osteogenic differentiation	38
3.5 Adipogenic differentiation	39
3.6 Chondrogenic differentiation	41
3.7 Gene expression profile	42

4. Discussion	45
4.1 Methods	45
4.1.1 Identification criteria	45
4.1.2 Sample preparation.....	45
4.1.3 Magnetic activated cell sorting (MACS).....	46
4.1.4 Flow cytometric analysis.....	47
4.1.5 Colony-forming unit (CFU) assay.....	49
4.1.6 Polymerase chain reaction (PCR)	50
4.1.7 Multilineage differentiation potential.....	50
4.2 Results	51
5. Summary	54
6. References	56
7. Appendix	68
7.1 Figures	68
7.2 Table	69
7.3 Abbreviations	70
7.4 Acknowledgment	72
7.5 Publications, Presentations and Patents derived from this thesis:	73
7.5.1 Peer review articles:	73
7.5.2 Citable abstracts:	73
7.5.3 Presentations:	73
7.5.4. Patents:	73
7.6 Curriculum vitae	75
7.7 List of publications	76

1. Introduction and review of literature

1.1 The periodontium

The periodontium is a highly cellular, vascular and specialized tissue. It comprises the root cementum, periodontal ligament (PDL), alveolar bone and gingiva. Principally, it functions to invest and support the tooth in the jaw. It further plays a crucial role in tooth function, homeostasis, and in repairing damaged tissue in response to mechanical, chemical or thermal trauma and in the event of a periodontal disease. Each of the periodontal components has its own very specialized structure, which directly define each of its tissue functions (Berkovitz 2004, Bosshardt 2005). It is becoming increasingly evident that each of them does not function in isolation, but they rather interact dynamically and influence each other in different manners. Correct physiologic functioning of the periodontium can only be attained through the preservation of the structural integrity as well as the complex relationships of its four components (Bartold et al. 2006, Nanci et al. 2006).

During embryogenesis the periodontium arises through a complex series of interactions between cells of the dental follicle, mainly derived from the multipotent neural crest ectomesenchyme. These neural crest cells, characteristic of vertebrates, sequester during fusion of the neural tube and subsequently migrate to the jaws where they participate in the formation of the dental follicle and dental papilla cells. After the formation of the dental organ, the epithelial root sheath of Hertwig arises from its apical extension, producing the root's prospective outline. On fragmentation of this epithelial root sheath, dental follicle cells come in contact with the newly formed root dentin. Upon this encounter, periodontal follicle cells start differentiating into various cellular types, including cementoblasts, fibroblasts and osteoblasts, laying down newly formed cementum on the freshly exposed root dentin, forming collagen fibres attachments (Sharpey's fibres) and finally anchoring these fibres into the surrounding newly formed cementum and alveolar bone, thereby giving rise to the periodontium, one of the body's most complex structures (Thesleff et al. 1995).

1.2 Periodontitis and periodontal regeneration

Periodontitis is a destructive inflammatory disorder of the periodontium, branded by the destruction of periodontal tissues namely the PDL, the cementum, the alveolar bone and the gingiva (American Academy of Periodontology, Glossary of Periodontal Terms; Bartold et al. 2006). Once these tissues are lost, the ideal, yet hard to achieve goal of periodontal

therapy is to regenerate the lost tissues if possible to their original form, architecture, and function. This is a demanding task that requires the harmonization of many actions at both the cellular and the molecular level.

Regeneration refers to the reproduction or reconstitution of a lost or injured part, in contrast to repair, which describes healing of a wound by tissues that does not fully restore the architecture or the function of the part (American Academy of Periodontology, Glossary of Periodontal Terms). Successful periodontal regeneration implies, histologically, the formation of new gingival connective tissue, the restoration of bone lost, and most decisively, the insertion of new connective tissue fibres into newly formed cementum on the formerly diseased root surfaces (Bartold et al. 2000). New attachment is defined as the union of connective tissue or epithelium with a root surface that has been deprived of its original attachment apparatus. This new attachment may be epithelial adhesion and/or connective tissue adaptation or attachment and may include new cementum. It is to be distinguished from reattachment, which describes the reunion of epithelial and connective tissue with a root surface not deprived of its fibrous attachment apparatus (American Academy of Periodontology, Glossary of Periodontal Terms). Bone fill is defined as the clinical restoration of bone tissue in a treated periodontal defect. Bone fill, however, does not address the presence or absence of histological evidence of new connective tissue attachment or the formation of a new PDL and can therefore not be taken solely, in case of a radiographic bone fill, as a proof of regeneration (American Academy of Periodontology 2005).

A variety of procedures to regenerate lost periodontal tissues have been suggested. One approach was based upon the introduction of a “filler” material into the periodontal defect in the hope of inducing bone regeneration. Materials used included autografts (cortical / cancellous bone, bone marrow), allografts (demineralized freeze-dried / freeze-dried bone), alloplastic materials (ceramics, hydroxyapatite, polymers and bioglass) and xenografts, but resulted with the exception of the auto- and partly the allografts in very little if any success (Bartold et al. 2000, American Academy of Periodontology 2005).

Autologous bone grafts of both extra- (iliac grafts) and intraoral (cortical and cancellous) sources have been used in periodontal regeneration attempts, relying on their cellular (e.g. osteoblasts) as well as growth factor (e.g. BMPs) content for a proposed osteogenic potential. Iliac grafts showed a mean bone fill ranging between 3.3 to 3.6 mm in intraosseous defects and a 2.5 mm increase in crestal bone height was further reported (Shallhorn et al. 1970). Intraoral cancellous bone with marrow grafts showed a mean bone fill ranging between 1.2 mm (Renvert et al. 1985) and 3.4 mm (Hiatt et al. 1973).

Regarding allografts, several types are currently commercially available from tissue banks. These include iliac cancellous bone and marrow, freeze-dried bone allografts (FDBA), and decalcified freeze-dried bone (DFDBA) allografts. Controlled clinical trials indicated bone fill ranging between 1.3 and 2.6 mm when FDBA were used to treat periodontal defects (Altieri et al. 1979, Rummelhart et al. 1989, Blumenthal et al. 1990). The use of DFDBA demonstrated bone fill similar to that achieved by FDBA, ranging from 1.7 to 2.9 mm (Mellonig 1984, Quientero et al. 1982, Rummelhart et al. 1989, Oreamuno et al. 1990). Controlled human histological studies with auto- as well as allograft material, using root notches into existing calculus as a reference point, demonstrated evidence of periodontal regeneration (American Academy of Periodontology 2005).

Concerning alloplasts, histological evaluation of treated teeth indicated limited if any regenerative potential, with minimal bone regeneration, fibrous encapsulation and no signs of new cementum or PDL regeneration (Shepard et al. 1986, Stahl et al. 1986). Finally, xenografts produced clinical results similar to other bone replacement grafts, though little if any periodontal regeneration can be expected with their use (American Academy of Periodontology 2005).

On the basis of the current understanding of the molecular and cellular biology of the periodontal development and regeneration, the simplistic approach of introducing a filler material into a periodontal bony defect is no longer plausible (American Academy of Periodontology 2005). In the event of alveolar bone formation, as with all the calcified structures in the body, a soft matrix is usually initially deposited by the forming cells, which eventually calcifies to form the final hard structure. The recommended simplistic procedure of an initial calcified filler material introduction pretty opposes this biological principal. Rather, efforts must be made to recapitulate in wound healing the crucial events that were associated with the original development and formation of the periodontium (Aukhil 2000, Bartold et al. 2000, 2006).

These new insights resulted later in the adoption of approaches based on the biological principles of tissue engineering. The first were the employment of various agents for root conditioning, either for demineralization of root surfaces with acids, for root surface coating with biological attachment agents such as fibronectin (Pearson et al. 1988), or both, to detoxify the roots as well as to create an environment suitable for cellular repopulation.

Root surface demineralization, usually with citric acid, has been employed as a part of regenerative procedures (Register et al. 1975, 1976). This technique was originally suggested because of the ability of citric acid to modify the root surface by “detoxifying” it (Daly 1982)

in addition to exposing collagen fibrils within the cementum or dentin matrix (Garett et al. 1978). Histological evaluation in human clinical trials demonstrated new connective tissue attachment and some regeneration following citric acid demineralization (Albair et al. 1982). However, results from other clinical trials indicated no additional improvement in clinical conditions when citric acid was applied in conjunction with surgical procedures, either in combination (Renvert et al. 1985) or without (Moore et al. 1987, Fuentes et al. 1993) osseous grafts. More recent studies indicated that the utilization of agents with a less acidic pH, such as ethylenediaminetetraacetic acid (EDTA), may expose collagen fibers, thereby promoting cellular attachment, without having any damaging effect on surrounding tissues (Blomlof et al. 1997). However, when tested in humans, this technique did not provide any significant clinical improvements (Caffesse et al. 1988). This conclusion was further confirmed by a meta-analysis systematic review, which stated that the use of citric acid, tetracycline, or EDTA to modify the root surface, provides no significant clinical benefit to regeneration in patients with chronic periodontitis (Mariotti 2003). In summary, histological evidence seemed to suggest that new connective tissue attachment and limited regeneration may result from root surface demineralization. Yet, this histological healing pattern did not result in significant improvement in clinical conditions beyond non-demineralized control sites (American Academy of Periodontology 2005). Furthermore, apart from not yielding a predictable regeneration outcome, these procedures often caused ankylosis and root resorption as side effects (Stahl et al. 1983, Caffesse et al. 1988, Kersten et al. 1992).

In the 1980s, guided tissue regeneration (GTR) was proposed as a new regenerative biologically based approach. Its idea relied on the introduction of a physical barrier, by surgically placing a membrane between the connective tissue of the periodontal flap and the curetted root surface. This membrane was expected to prevent apical migration of gingival epithelial and connective cells onto the root surface and to thereby facilitate the repopulation of the periodontal wound site exclusively with the desired PDL cells. The approach was thought to bring about regeneration instead of the commonly undesired long junctional epithelial healing (Nyman et al. 1982). Guided tissue regeneration was reported to be consistently more effective than open flap debridement in terms of gain in clinical attachment level and probing depth reduction in the treatment of periodontal intrabony and furcation defects (Pontoriero et al. 1989). Several barrier materials have been employed in GTR studies, including both non-resorbable and bioabsorbable (collagen, polylactic acid and connective tissue) membranes. Although no significant differences were detected among barrier types, the different barrier types could account for some of the widely inconsistent

results (Pontoriero et al. 1989). Yet, long-term studies and evaluations of this method indicated that, although this method resulted in true regeneration, the clinical improvements obtained by this procedure were of small magnitude and exhibited large variability (Bartold et al. 2000, Murphy et al. 2003).

More recently, the application of a variety of growth and differentiation factors to stimulate cellular division and repopulation of periodontal defects and, subsequently, regeneration has been investigated. Various growth factors were in the past and are currently under in vitro and in vivo investigation, comprising platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), bone morphogenetic proteins (BMPs) and enamel matrix proteins, in the hope to bring about the differential growth, repopulation of the root surface with certain types of tissues and cells and finally differentiation of these cell populations along certain pathways. However, the numbers of well-controlled long-term studies (Ripamonti et al. 1994, American Academy of Periodontology 1996, Giannobile et al. 2003) remain insufficient to deduce any substantial conclusions about the clinical utility of growth factor application in human periodontal tissue regeneration. While these growth and differentiation factors certainly show some promise, it is possible that they, too, will be less than satisfactory (Bartold et al. 2000).

From the different approaches through these years it has, however, been learned that the local environment with its extracellular matrix and molecules is a crucial element for the differentiation of the regenerating cells towards the desired tissues and that healing site protection from the oral environment is of utmost importance for successful periodontal regeneration. It has become further clear that the way forward in the field of periodontal regeneration is certainly through the application of current knowledge in the fields of molecular and cellular biology, developmental biology and tissue engineering principles as applied to tissue regeneration and that in order for periodontal regeneration to occur, many events have to be guided and regulated at these cellular and molecular levels.

It appeared that one of the most important factors hampering the current regeneration attempts was still the availability of a sufficient number of progenitor cells to carry out the regeneration and differentiation processes, as has been previously addressed by Melcher (1985) in his work. 25 years ago, Melcher proposed his compartmentalization model stating that PDL fibroblasts were the cells most important in the regeneration process and questioned whether the three cell populations of the periodontium (cementoblasts, alveolar bone cells and PDL fibroblasts) were ultimately derived from a single population of ancestral or progenitor cells.

The idea then emerged that since periodontal regeneration should be ideally a re-enactment of the original periodontal development process including cytodifferentiation, morphogenesis, extracellular matrix production and mineralization, such processes would require the presence of sufficient number of progenitor cells to possibly differentiate into cementoblasts, osteoblasts and fibroblasts. So if in the event of injury to the periodontium these potential progenitor cells were present in a satisfactory number, they could be activated towards terminal differentiation and regeneration would occur (Bartold et al. 2006).

To achieve these goals, different stem cell sources as well as methods for isolation, ex-vivo expansion and characterization of these potential progenitor cells needed to be developed and are currently extensively investigated.

1.3 Embryonic and adult stem cells

Stem cells are progenitors characterized by their ability for self-renewal through mitotic cell division and by an inherent capability to subsequently differentiate into a diverse range of specialized cell types under suitable conditions (Wagers et al. 2004, Cai et al. 2004, Robey et al. 2006, Morsczeck et al. 2008).

There are two main subdivisions of stem cells, the embryonic and the adult types. Embryonic stem cells originate from the mammalian blastocyst during early embryonic development and are postulated to be pluripotent, having the ability to give rise to every specialized tissue belonging to the three different germ layers: mesoderm, ectoderm and endoderm.

However, the isolation and use of these stem cells is technically difficult besides being ethically highly controversial. A further problem is the immortal nature of these stem cells, based on their high telomerase expression. This feature poses an additional risk factor, bearing with it the undesired possibility of an eventual carcinogenic transformation of the transplanted stem cells. All these aspects hampered the wide use and application of embryonic stem cells in human regenerative therapies.

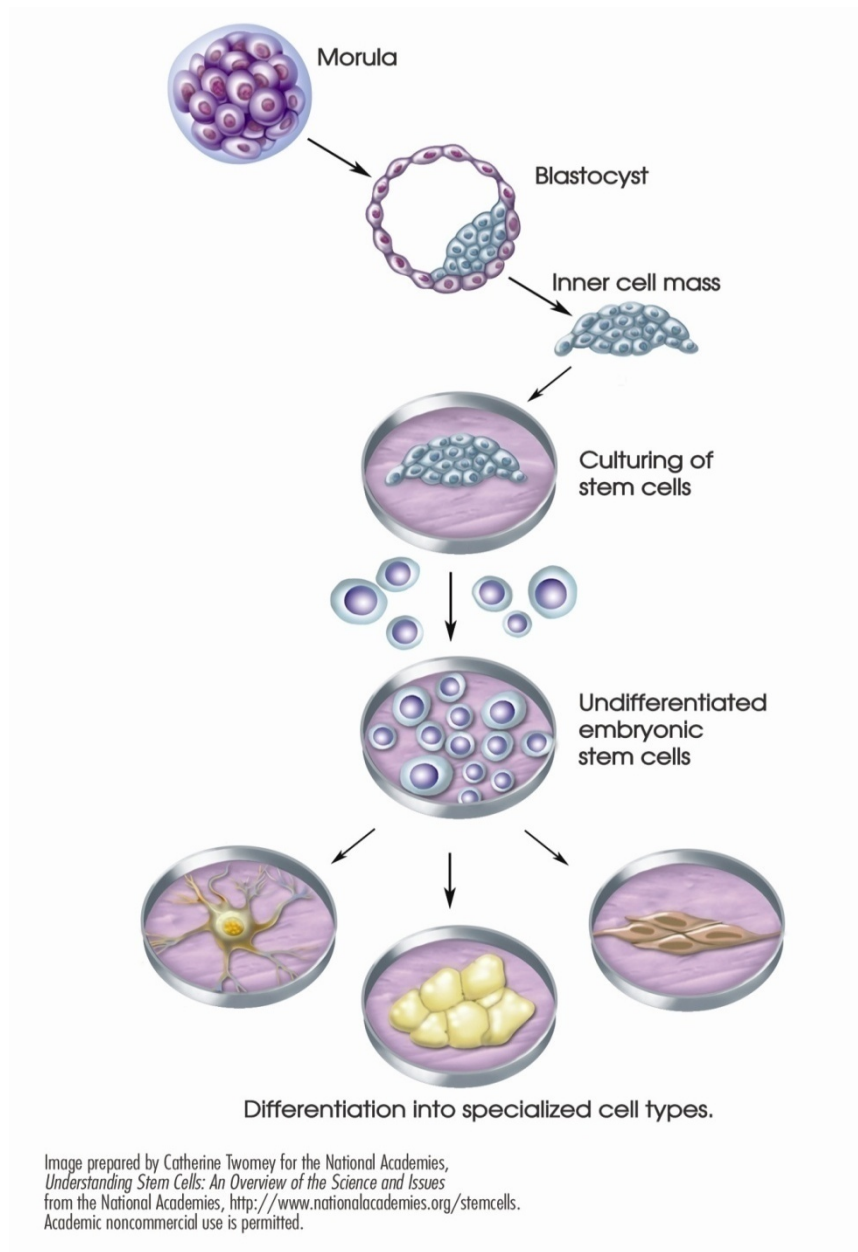


Figure 1: Embryonic stem cells

Yet, adult or postnatal stem cells, also known as somatic stem cells, existing in many organs of the human body pose a readily accessible source and are, compared to embryonic ones, less problematic in ethical terms (Gronthos et al. 2006, Maunon et al.2007, McAllister et al. 2009). They have been discovered in numerous organs that are characterized by a high turnover rate and therefore need a steady supply of cells, such as the blood, skin, and lining of the gut, and have also been established in unanticipated organs as the brain, which is an organ usually branded by its inability to replace any of the damaged cells in case of injury.

Postnatal or adult stem cells can be subdivided into hematopoietic and mesenchymal types and are both defined by two major properties: self-renewal and multipotency (Fischbach et al 2004). Self-renewal is defined as the ability of asymmetric cell division, where one daughter cell remains a stem cell while the other becomes a committed progenitor cell. This is an important property for any successful use of stem cells in therapy, ensuring a constant supply of cells. Multipotency is a unique property of adult stem cells. Under suitable conditions, adult stem cells have a wide differentiation potential with the capability to develop into many cell types in the body. If this differentiation could be controlled and sustained, adult stem cell therapies could become the basis of treatment for various severe diseases and injuries.

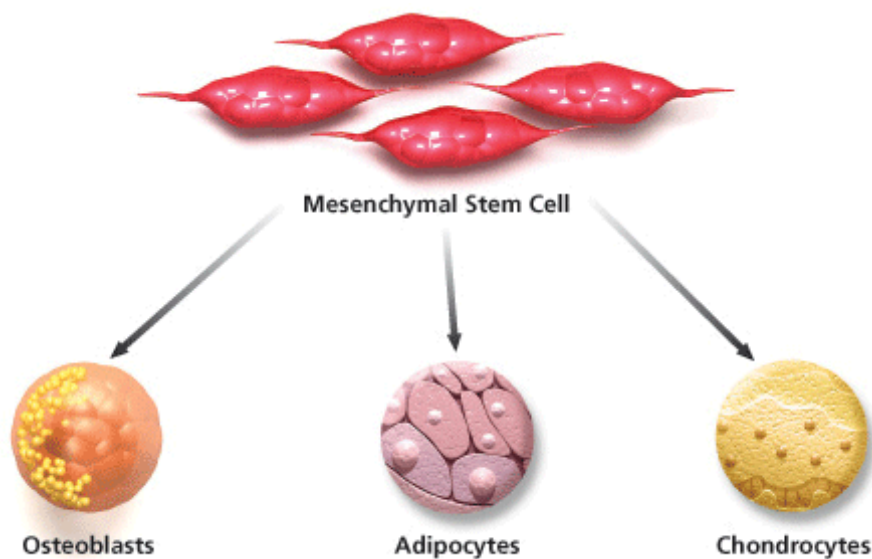


Image from: <http://www.sigmaaldrich.com/life-science/stem-cell-biology/mesenchymal-stem-cells.html>

Figure 2: Mesenchymal stem cells

The history of research on adult stem cells began about 60 years ago. In the 1950s, scientists discovered that the bone marrow contains at least two populations of stem cells; the hematopoietic stem cells, competent of forming all types of blood cells in the body and, a few years later, the bone marrow stromal (mesenchymal) stem cells making up a small proportion of the stromal cell population in the bone marrow and capable of generating bone, cartilage, fat and fibrous connective tissue. In the 1960s, researchers studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells (Cooper-Kuhn et al. 2002). Despite these reports, most scientists continued to believe that the adult

brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does harbor stem cells, that have the ability to generate the brain's major cell types, astrocytes and oligodendrocytes, which are non-neuronal cells, as well as neurons, or nerve cells. But although research on adult stem cells has begun decades ago, significant novel breakthroughs have been made only in the past few years.

Adult stem cells have many essential advantages making them promising therapeutic alternatives. On the one hand, since adult stem cells are obtained from the patient's own tissues, they are genetically matched and would therefore not be rejected by that patient's own immune system or elicit any possible immune reaction. On the other hand, they are far less controversial in ethical terms as compared to embryonic stem cells. Their finite life span also limits the potential of carcinogenic transformation as compared to embryonic ones, branded by a high telomerase expression. Their multipotent differentiation potential remains of course one of their best merits, giving hope for the cellular approach concept in tissue regeneration. These aspects are responsible of the very promising success in treating many diseases and injuries in clinical trials using adult stem cells, such as leukemia, lymphoma, diabetes, advanced kidney cancer and several inherited blood disorders.

With the emerging concept of cellular therapy in tackling many diseases and disorders with no previously predictably successful treatment outcomes, periodontitis came into the spot light. Many different types of stem cells isolated from both oral as well as extra oral tissue in the past years have been implemented in order to regenerate tissues lost due to periodontal disease under the umbrella of the developing field of tissue engineering. Many modifications have been introduced in many aspects of this field throughout the course of the development of this treatment concept, from scaffold inventing, to scaffolds modification, to growth factor and cytokine utilization and, finally, to cellular modification using special transfection procedures, trying to optimize the regenerative outcome.

One of the directions of optimization in the area of stem cell therapy was that of determining the ideal stem cell source for periodontal regenerative approaches, where on one hand the cells should be multipotent and capable of regenerating all lost tissue types while being accessible with minimal morbidity and mortality to the subject and on the other hand the tissues serving as a supply for the isolation of these cells should pose an easily and readily accessible and available source. Recently, dental tissues such as the PDL, the dental pulp, the dental papilla and the tooth follicle have been recognized as readily available sources for adult stem cells (McAllister et al. 2009).

1.4 Types of adult stem cells currently investigated in the dental field

In the field of tooth engineering and periodontal regeneration, efforts have been made to isolate and investigate the potential of adult mesenchymal stem cells (MSCs) from multiple cell sources such as bone marrow stromal stem cells (BMSSCs), adipose derived stromal cells (ADSCs), adult dental pulp stem cells (DPSCs), stem cells from the dental follicle (DFSCs), stem cells from human exfoliated deciduous teeth (SHEDs) and periodontal ligament stem cells (PDLSCs) (Bluteau et al. 2008). The following section is intended to review the current state of knowledge and dental use of these different types.

1.4.1 Bone marrow stromal stem cells (BMSSCs)

Circulating blood cells such as erythrocytes, platelets, monocytes, granulocytes, and lymphocytes normally survive for only a few days or months in the peripheral tissues. Their continuous supply is constantly ensured by hematopoietic progenitors or stem cells (HSCs) in the bone marrow. However, beside these HSCs the bone marrow also contains cells that meet the criteria for stem cells of nonhematopoietic tissues called bone marrow stromal stem cells (BMSSCs). BMSSCs are bone marrow cell populations that can be easily isolated using their plastic adherence property and are capable of differentiating along multiple mesenchymal lineages into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells, or nerve cells in vitro and in vivo (Bruder et al. 1997, Pittenger et al. 1999, Makino et al. 1999, Kopen et al. 1999, Deans et al. 2000, Kuznetsov et al. 2001, Tsutsumi et al. 2001).

From the point of tooth and tissue engineering, trials in the fields of tooth-like structures formation and periodontal regeneration employing BMSSCs were attempted. BMSSCs cultures were usually composed of a mixture of fibroblasts, osteoblasts, adipocyte progenitors and up to 4-19% stem cells (Pereira et al. 1998, Pittenger et al. 1999). Although most tooth engineering researches were originally attempted using purified cell populations of these stem cells, Ohazama et al. (2004) gave evidence that bone and soft tissues could also be formed from a heterogeneous population of bone marrow derived cells. In their study tooth structures were formed by transferring intact explants from bone marrow derived cells into renal capsules. They further combined embryonic oral epithelium with three types of stem cells such as embryonic stem cells, neural stem cells and adult bone marrow derived cells, and transferred the recombination into adult renal capsules and adult jaw, forming tooth-like structures and bone. In 2007, a study conducted by Li et al. came to a conclusion in concert with them. They stated that the combination of BMSSCs with oral epithelial cells from rat

embryos resulted in their expression of odontogenic genes such as Pax9, dentine matrix protein 1 (DMP1), and dentine sialophosphoprotein (DSPP) and formed tooth-like structures histologically.

BMSSCs have also been tested for their ability to regenerate periodontal tissue. The auto-transplantation of BMSSCs in an animal study was postulated to be able to histologically regenerate periodontal defects almost completely with cementum, PDL and alveolar bone in four weeks, providing a competitive source of MSCs for the treatment of periodontal diseases (Kawaguchi et al. 2004). Hu et al. (2006b) investigated the possibility of BMSSCs giving rise to ameloblasts, and showed that BMSSCs can be reprogrammed to give rise to ameloblast-like cells. This offered BMSSCs a novel perspective for tooth-tissue engineering having the potential to be induced into both mesenchymal and epithelial directions.

Still, Jing et al. (2008) pointed out that the major limitation in their application remained the differentiation capabilities of BMSSCs, which were strongly age dependent, decreasing considerably with increasing donor age.

1.4.2 Adipose-derived stromal cells (ADSCs)

Studies conducted on adipose-derived stromal cells (ADSCs) affirmed that ADSCs contained a group of pluripotent mesenchymal stem cells with stable growth and proliferation kinetics *in vitro*. They could be obtained by less invasive methods and in larger quantities than BMSSCs, making their use as a potential source of stem cells very attractive (Zuk et al. 2002).

ADSCs manifested multilineage differentiation capacities into osteogenic, chondrogenic and adipogenic directions (Liu et al. 2008). Hicok et al. (2004) stated that the ability of ADSCs to form osteoid matrix *in vivo* could pave the way for novel therapeutic approaches in bone repair and regeneration. Kakudo et al. (2008) investigated the possibility of using a honeycomb collagen scaffold to culture ADSCs in bone tissue engineering and showed that the scaffold was filled with the grown ADSCs and calcification materials. When the ADSCs-loaded honeycomb collagen scaffolds were transplanted into nude mice, bone formation was identified after eight weeks.

On the gene level ADSCs impressively expressed many important bone marker proteins including alkaline phosphatase (ALP), type I collagen, osteopontin, and osteocalcin (Wu L et al. 2008).

In 2008 it was first proposed that ADSCs could be induced into the odontogenic lineage and might be suitable for seeding cells for tooth regeneration as an alternative to replace lost teeth

in elderly patients (Jing et al. 2008). Jing et al. further claimed that the commonly employed cells for tooth regeneration such as odontoblasts from dental germ, stem cells from dental pulp and deciduous teeth, and ectomesenchymal cells from the first branchial arch are difficult, or even impossible, to harvest clinically. BMSSCs on the other hand, although having odontogenic capacity, showed a major disadvantage through a significant decrease in their differentiation abilities with increasing age of the donors and are therefore in their view not clinically practical in tooth regeneration in donors of old age. In this aspect, ADSCs would represent a very promising cellular alternative. However, the need to perform the demanding task of harvesting these cells from an extra-oral adipose tissue source by a dentist could make them not the ideal stem cell source in dental practice.

1.4.3 Adult dental pulp stem cells (DPSCs)

Although the regenerative capacity of the human dentine/pulp complex is not well understood, it is clinically evident that upon pulpal injury reparative dentine forms as a protective barrier for the pulp complex (Murray et al., 2001). The natural regenerative capacity of the human dentine/pulp complex points at the possibility that dental pulp may contain progenitors responsible for dentine repair.

Gronthos et al. (2000) first identified adult dental pulp stem cells (DPSCs) in human dental pulp. Shi et al. (2001) demonstrated that DPSCs showed a higher proliferation rate compared to BMSSCs cultured under the same conditions, which could be related to their strong expression of the cell-cycle activator, cyclin-dependent kinase 6.

The expression of various different perivascular cell markers, such as STRO-1, VCAM-1, CD146/MUC-18 and α -smooth-muscle actin by these cells provides evidence that DPSCs could represent a heterogeneous population of MSCs and that they are likely located in the perivascular niches within the dental pulp complex (Gronthos et al. 2000, Shi and Gronthos 2003).

In 2003 Shi and Gronthos demonstrated that DPSCs could produce a dentine-pulp-like structure, composed of mineralized matrix with tubules lined with odontoblasts and fibrous tissue containing blood vessels, similar to the arrangement found in the ordinary dentine-pulp complex of human teeth. Many studies also showed that DPSCs possess a self-renewal capability and multilineage differentiation potential into odontoblasts, chondrocytes, adipocytes, and neural-like cells under appropriate environmental conditions (Gronthos et al. 2002, Iohara et al. 2006, Hosoya et al. 2007). An *in vivo* study affirmed that DPSCs produced bone after implantation into subcutaneous sites in immunocompromised mice employing a

hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold. In addition, it was found that even after two years of storage, DPSCs were still able to differentiate into pre-osteoblasts and form woven bone, and expressed certain surface antigens, confirming cellular integrity (Papaccio et al. 2006, Otaki et al. 2007).

Yet, in a study by Zhang et al. (2006) DPSCs seeded onto different 3-dimensional scaffold materials, namely spongy collagen, porous ceramic and fibrous titanium mesh, and implanted in nude mice for six or twelve weeks failed to form a dentine- pulp-like complex but rather differentiated into a tissue that more resembled a connective tissue. This finding together with contrasting earlier reports that DPSCs produced a dentine-pulp-like structure, composed of mineralized matrix with tubules lined with odontoblasts and fibrous tissue containing blood vessels similar to the arrangement found in the ordinary dentine-pulp complex of human teeth (Shi and Gronthos 2003), did not make these cells the ideal stem cell source to be employed in periodontal regeneration attempts.

1.4.4 Dental follicle stem cells (DFSCs)

The dental follicle is the mesenchymal component that surrounds the developing tooth germ. During tooth root formation the cementum, PDL and alveolar bone are derived from the interactions within the dental follicle (Yokoi et al. 2007). Stem cells from dental follicle or dental follicle stem cells (DFSCs) have been isolated from the follicle of human impacted third molars and were found to express the stem cell markers Notch1, STRO-1, and nestin (Morsczech et al. 2005). In 2007 Luan et al. indicated that DFSCs cell lines were heterogeneous and consisted of three main lineages: a highly undifferentiated state of periodontal ligament cell-type lineage, a cementoblastic, and an osteoblastic lineage.

DFSCs, similar to DPSCs, were found to possess the ability to differentiate into osteoblasts/cementoblasts, adipocytes, and neurons (Kémoun et al. 2007, Yao et al. 2008, Coura et al. 2008). In addition, when DFSCs were transplanted into immunodeficient mice they were reported to recreate PDL-like tissue after four weeks (Yokoi et al. 2007).

Wu J et al. (2008) verified that dentine non-collagenous proteins (dNCPs) extracted from dentine stimulated DFSCs to differentiate into a cementoblast lineage. In 2007 Kémoun et al. demonstrated that enamel matrix derivatives (EMD) solely or in combination with BMP-2/-7 guided human dental follicle stem cells (hDFSCs) towards a cementoblastic phenotype. Tsuchiya et al. (2008) also reported that the interaction between DFSCs and collagen type I facilitated their differentiation along the mineralization process.

Compared to DPSCs, DFSCs have a greater number of STRO-1 positive cells, a faster proliferation rate, a greater number of population doublings and increased capacity for in vivo dentine regeneration. However, unlike DPSCs and other MSCs, DFSCs are positive for telomerase activity, a characteristic of embryonic stem cells that principally confers immortality (Sonoyama et al. 2006, 2008, Yang et al. 2008). Whether this expression poses an advantage or a potential risk factor, similar to the one posed by embryonic stem cells, in tissue engineering needs still to be clarified.

1.4.5 Stem cells from human exfoliated deciduous teeth (SHEDs)

An exfoliated deciduous tooth typically contains living pulp remnants harbouring stem cells known as stem cells from human exfoliated deciduous tooth (SHEDs). They remain alive inside the tooth for a very short time after its exfoliation, during which they can be harvested. The discovery of SHEDs by Miura et al. (2003) pointed at the interesting possibility of using them for tissue engineering, especially in regard to the ease of accessibility to human exfoliated deciduous teeth (Murray and Garcia-Godoy 2004, Sloan and Smith 2007).

The reported advantages of SHEDs were their higher proliferation rate compared with stem cells from permanent teeth, their ease of expansion in vitro, their high plasticity with the ability to differentiate them into neurons, adipocytes, osteoblasts, and odontoblasts, and their good accessibility in the young with little or no morbidity to the patient (Miura et al. 2003).

Ex vivo expanded SHEDs expressed STRO-1 and CD146 (MUC18), the two early cell surface markers for BMSSCs (Shi and Gronthos 2003). In addition, SHEDs expressed a variety of osteoblastic/odontoblastic markers, including Runx2, ALP, matrix extracellular phosphoglycoprotein, bone sialoprotein (BSP), and DSPP. SHEDs isolated from exfoliated deciduous incisors also formed adherent clusters similar to other MSCs in vitro and exhibited a higher rate of proliferation when compared to BMSSCs and DPSCs (Miura et al. 2003, Koyama et al. 2009).

Regarding their osteogenic potential, Miura et al. (2003) stated that SHEDs could not differentiate directly into osteoblasts, but had distinctive osteoinductive abilities, inducing new bone formation by recruiting host osteogenic cells. In contrast, Cordeiro et al. (2008) in their study showed that when SHEDs seeded in poly-L-lactide acid (PLLA) scaffolds were transplanted into the subcutaneous tissue of immunodeficient mice, they differentiated into odontoblast-like cells, showed morphologic characteristics resembling odontoblasts, and an increase in microvascular density was noted in the implant surrounding. They also verified that the transplanted SHEDs were capable of differentiating into blood vessels that

anastomosed with the host vasculature ensuring a vital and continuous vascular supply to the newly implanted construct.

Whether SHEDs demonstrate the same shortcomings of DPSCs, being both derived from the dental pulp of the teeth, is still to be clarified. Another point to be illuminated is whether these cells really represent a stem cell population different from DPSCs.

1.4.6 Periodontal ligament stem cells (PDLSCs)

The periodontium is one of the most highly specialized and complex connective tissues of the human body. It is derived from dental follicle, which in turn originates from the neural crest cells. It is well established that the PDL contains a heterogeneous population of progenitor cells (Lekic et al. 2001, Murakami et al. 2003). These cells are thought to be responsible for maintaining tissue homeostasis and to play a crucial role in periodontal regeneration (Beertsen et al. 1997). A study by Seo et al. in 2004 identified and characterized human PDL-derived mesenchymal stem cells as periodontal ligament stem cells (PDLSCs).

PDLSCs are reported to have a higher proliferation rate than BMSSCs, with a longer lifespan and a higher number of population doublings in vitro. PDLSCs cultures demonstrated approximately 30% higher rates of proliferation compared to cultured BMSSCs and it appeared that PDLSCs retain this capacity of higher growth potential beyond 100 population doublings before in vitro senescence is noted, compared to approximately 50 population doublings for BMSSCs (Bartold et al. 2006).

Another study has shown that mesenchymal stem cells obtained from the PDL are multipotent cells with features similar to BMSSCs and DPSCs, having the ability to differentiate into adipocytes, cementoblast-like cells, and collagen forming cells (Seo et al. 2004). PDLSCs in the presence of inductive media containing ascorbic acid, dexamethasone and an excess of inorganic phosphate form Alizarin Red-positive mineralized deposits in vitro. However, although they formed mineralized nodules with calcium accumulation in vitro, this nodule formation was lower when compared to DPSCs (Seo et al. 2004). Orciani et al. (2009) also demonstrated the osteogenic ability of PDLSCs and emphasized that differentiating cells were also marked by an increase in Ca^{2+} and nitric oxide production and that the local re-implantation of expanded cells together with a nitric oxide donor could be a promising scheme for regeneration of periodontal defects.

Beside their osteogenic ability, the cementoblastic potential of PDLSCs was further demonstrated (Isaka et al. 2001). A conditioned medium from developing apical tooth germ cells (APTG-CM) was shown to be capable of providing the cementogenic micro-

environment necessary to encourage the differentiation of PDLSCs to the cementoblastic lineage (Yang et al. 2008). dNCPs were also shown to increase proliferation and adhesion aptitude of PDLSCs. The induced PDLSCs demonstrated several features of cementoblast differentiation (Ma et al. 2008). In the presence of adipogenic inductive media PDLSCs further formed Oil Red O-positive deposits (Seo et al. 2004).

Notably, studies on PDLSCs isolated from root surfaces of extracted teeth have demonstrated their competence for tissue regeneration and periodontal repair (Seo et al. 2004, Kim et al. 2007, Liu et al. 2008). In the study by Seo et al. (2004), human PDLSCs were incorporated into a HA/TCP scaffold and implanted subcutaneously into immunocompromised mice, resulting in a cementum with PDL-like structure being formed. When the same cells were transplanted into surgically created defects in periodontal areas in mandibular molars in immunocompromised mice, these cells were reported to integrate into the PDL and to attach to the surrounding alveolar bone and cementum surfaces. In addition, these transplants formed a type I collagen-positive PDL-like tissue, morphologically similar to Sharpey's fibres, connecting with the newly formed cementum. Using human-specific antimitochondria antibodies, the cells responsible for the regeneration of these tissues have been clearly identified as being of human origin.

This distinctive ability of PDLSCs to generate a cementum and PDL-like tissue *in vivo* (Seo et al. 2004), in contrast to the dentine or pulp-like structure and lamellar bone and marrow-like structure, generated by DPSCs and BMSSCs, respectively (Kuznetsov et al. 1997, Gronthos et al. 2000, Gronthos et al. 2003a) clearly demonstrated that PDLSCs represent a unique mesenchymal stem cell population ideal for periodontal regenerative stem cell therapies (Coura et al. 2008).

Bearing this in mind, the exploitation of the potential periodontal stem cells to produce functional periodontal tissues represents a promising therapeutic option for regenerating periodontal tissue under the umbrella of the developing field of tissue engineering (Stock et al. 2001, Griffith et al. 2002, Bartold et al. 2006).

1.5 Identification criteria for PDLSCs

For identifying PDLSCs many criteria have been proposed. PDLSCs usually generate characteristic clonogenic clusters of adherent fibroblastic-like cells or fibroblastic colony-forming units when cultured under the same growth conditions described for bone marrow stromal stem cells (Seo et al. 2004). Noteworthy, the frequency of fibroblastic colony-

forming units (aggregates of 50 cells or more) derived from PDL was higher than that noted for BMSSCs (170 for PDLSCs and 14 for BMSSCs per 10^5 cells plated).

The stem cell marker STRO-1, used to isolate and purify BMSSCs, is also expressed by PDLSCs and DPSCs and was commonly employed to isolate human PDLSCs using immunomagnetic or fluorescence-activated cell sorting. In addition, PDLSCs also share in common with BMSSCs and DPSCs the expression of the perivascular cell marker CD146/MUC18 (Seo et al. 2005). A proportion of PDLSCs also co-expressed alpha-smooth muscle actin and/or the pericyte-associated antigen 3G5 (Bartold et al. 2006). These observations entailed a perivascular origin for these cells consistent with the results of McCulloch et al. 1987, who demonstrated the occurrence of progenitor cells within the perivascular spaces of mouse PDL. Both PDLSCs and BMSSCs were negative for the hematopoietic markers CD14, CD45, and CD34 (Bartold et al. 2006).

On the gene level, mature mineralized tissue markers, such as ALP, type I and III collagens, osteonectin, osteopontin, osteocalcin, and BSP were expressed by these cells (Ivanovski et al. 2001, Shi et al. 2001, Gronthos et al. 2003b, Seo 2004). Moreover, PDLSCs expressed high levels of scleraxis, a tendon-specific transcription factor associated with tendon cells (Seo et al. 2004, Nagatomo et al. 2006).

Interestingly, DPSCs, SHEDs, and PDLSCs appear to have similar gene expression profiles for extracellular matrix proteins, growth factors receptors and adhesion molecules, attesting to the existence of an universal origin and molecular pathway regulating the formation of dentine, cementum, and bone. To date, no genes are exclusively expressed by either cell population (Shi & Gronthos 2003, Shi et al. 2005).

1.6 Criteria for the identification of MSCs

In 2006, a position paper published by the International Society for Cellular Therapy defined the standards accepted to identify human MSCs, which they renamed multipotent stromal cells, phenotypically and functionally (Dominici et al. 2006). Firstly, the alleged MSCs must be plastic-adherent when maintained under standard culture conditions. Secondly, more than 95% of the alleged MSCs' population must express the surface markers CD73, CD90, and CD105 as measured by flow cytometry and these cells must lack the expression (less than 2%) of the surface markers CD45 (also called common leukocyte antigen), CD34 (normally present on hematopoietic stem and progenitor cells as well as on the endothelium), CD14 (normally present on monocytes and macrophages). Thirdly, these cells must be able to

differentiate into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions, as demonstrated by standard staining protocols.

1.7 Aim of the thesis

The aim of the current study was to isolate and characterize potential multipotent postnatal periodontal stem cell populations (PDSCs) from the gingival cervical margin as well as the alveolar bone, adhering strictly to both the criteria for the identification of periodontal as well as mesenchymal stem cells.

As all components of the periodontium share a common developmental pathway and regarding the fact that PDLSCs have been isolated by Seo et al. (2004) from the PDL, it was the hypothesis of this study that the gingival margin as well as the alveolar bone could harbour similar multipotent periodontal mesenchymal stem cell populations.

In order to enrich potential stem cells for the in vitro culture, the study will start by isolating these cells by magnetic sorting. It will be then asked whether the isolated PDSCs possess a colony forming ability, the defined surface marker composition for mesenchymal stem cells, an osteogenic marker expression profile, and, finally, multipotent characteristics, making them competitive candidates for any prospective cellular therapy in the field of periodontal regeneration.

2. Material and Methods

2.1 Sample size and characteristics

The current study was approved by the ethical committee of the Christian-Albrechts-University, Kiel. After obtaining the patients' informed consent, eleven healthy third molars from eight different individuals (four males and four females) were extracted. All extracted teeth had an alveolar bone part as well as a cervical gingival soft tissue part attached to their root surfaces.

The samples were placed immediately after extraction in 50 ml sterile polypropylene tube containing Minimum Essential Medium Eagle Alpha Modification (Sigma-Aldrich, Hamburg, Germany) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and 1% amphotericine (all from Biochrom AG, Berlin, Germany).

2.2 Cell isolation and cultivation protocols

In this study two protocols were developed to isolate and culture different tissues to obtain PDSCs:

2.2.1 1st Protocol

Under the laminar flow hood, alveolar bone parts attached to the root surface were detached and cut into small pieces. The separated bone spicules were immersed immediately and rinsed several times in Minimum Essential Medium Eagle Alpha Modification supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine (Biochrom AG). The resultant bone spicules were inserted into 25 ml tissue culture flasks (Sarstedt AG, Nümbrecht, Germany) and left to adhere to their bottoms. Afterwards, Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% fetal calf serum (FCS) (HyClone, Logan, UT, USA), 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine was carefully added taking care not to detach the adherent bone spicules. The cell culture flasks were incubated in a 5% CO₂ incubator (Heraeus, Hanau, Germany) at 37°C. Cells were left to adhere and grow out of the bone spicules for one week before changing the medium for the first time and discarding the spicules. The flasks were periodically checked every 24 hours by a phase contrast inverted

microscope. The culture medium was changed three times per week until the cells reached about 80-85% confluence.

2.2.2 2nd Protocol

Under the laminar flow hood, soft tissue collar attached at the cervical margin of the teeth were detached and kept as much as possible intact. The separated soft tissue masses were de-epithelised and immersed immediately and rinsed several times in Minimum Essential Medium Eagle Alpha Modification supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine. The resultant soft tissue masses were inserted into 25 ml tissue culture flasks and left to adhere to their bottoms. Afterwards Minimum Essential Medium Eagle Alpha Modification supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine was carefully added to the culture flasks, taking care not to detach the adherent tissue masses. The cell culture flasks were incubated in a 5% CO₂ incubator at 37°C. Cells were left to adhere and grow out of the soft tissue masses for one week before changing the medium for the first time and discarding the soft tissue masses. The flasks were periodically checked every 24 hours by a phase contrast inverted microscope. The culture medium was changed three times per week until the cells reached about 80-85% confluence.

2.3 Cell Passage

After the cells cultured from both protocols have reached 80-85% confluence they were sub-cultured as follows:

Cells in the flasks were washed twice with 5 ml phosphate buffered saline (PBS) (Biochrom). Afterwards 2 ml of 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) (Biochrom) was added and the flasks incubated in a 5% carbon dioxide incubator at 37°C for 5 min to detach the cells from the flasks' bottom. To inactivate trypsin, 5 ml of the culture medium containing FCS was added. The cell suspensions were transferred to a sterile Falcon tube (Sarstedt) and centrifuged at 2000 rpm for 10 min. After discarding the supernatant, cells were resuspended in 2 ml of Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine. Cells were counted and viability tested using Trypan blue (Sigma-Aldrich) to be finally seeded at a density of 30 cells/cm² in 75 ml culture flasks. 10 ml of culture medium was added to each culture flask. The flasks were incubated in

5% CO₂ incubator at 37°C and cells left to adhere and grow. Again, the culture medium was changed three times per week.

2.4 Magnetic activated cell sorting (MACS)

After the first passage cells have reached 80-85% confluence, they were subjected to magnetic activated cell sorting (MACS). Following aspiration of the medium, cells were washed twice with PBS. 2-3ml 0.05% trypsin-0.02% EDTA diluted 1:10 was added to each culture flask and the flasks were incubated in 5% CO₂ incubator at 37°C for 5 min. 3 ml medium (with FCS) was added to each flask to deactivate the trypsin. Detached cells were transferred to new Falcon tubes and centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the resultant cell pellet resuspended in 10 ml of fresh medium. A cell count was performed, the cell suspension centrifuged for 5 min at 1500 rpm and the supernatant discarded.

2.4.1 MACS Method

The magnetic field was disinfected with ethanol, the apparatus assembled and the column attached to the magnetic field according to manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). The column was rinsed with 500 µl MACS-buffer consisting of 0.5 % bovine serum albumin (BSA) and 2 mM EDTA in PBS (all from Biochrom). Two tubes were prepared to be put below the column; one for the positively and the other one for the negatively MACS-sorted fractions.

After incubating the cells with r FcR- blocking reagent (Miltenyi Biotec) for 5 min, STRO-1 (BioLegend, San Diego, CA) and anti IgM MicroBead antibodies (Miltenyi Biotec) were added to the cells according to manufacturer's instructions for 15 min at 4 °C. Ten times MACS-buffer volume was added to the cell suspension and the suspension centrifuged for 10 min at 1700 rpm at 4 °C. The supernatant was discarded and the resultant cell pellet resuspended in 500 µl MACS-buffer (cells < 10⁸) or 1000 µl MACS-buffer (cells > 10⁸).

Cells were transferred to the column and the tube for the negative fraction set below it. The column was washed three times with 500 µl MACS-buffer. Afterward the column was taken off the magnetic field, the positive-fraction cells washed out with 1 ml MACS-buffer and pressed out with a plunger in a sterile tube (tube for positive fraction). The positive-fraction cells were filled with MACS-buffer to a volume of 5 ml. A second cell count was performed while the cell suspension was centrifuged for 10 min at 1700 rpm at 4 °C. The supernatant

was discarded, the cells resuspended in the culture medium, a fraction of the cells seeded in new 75 ml culture flasks at a concentration of 30 cells/ cm² and a fraction subjected to flow cytometric analysis.

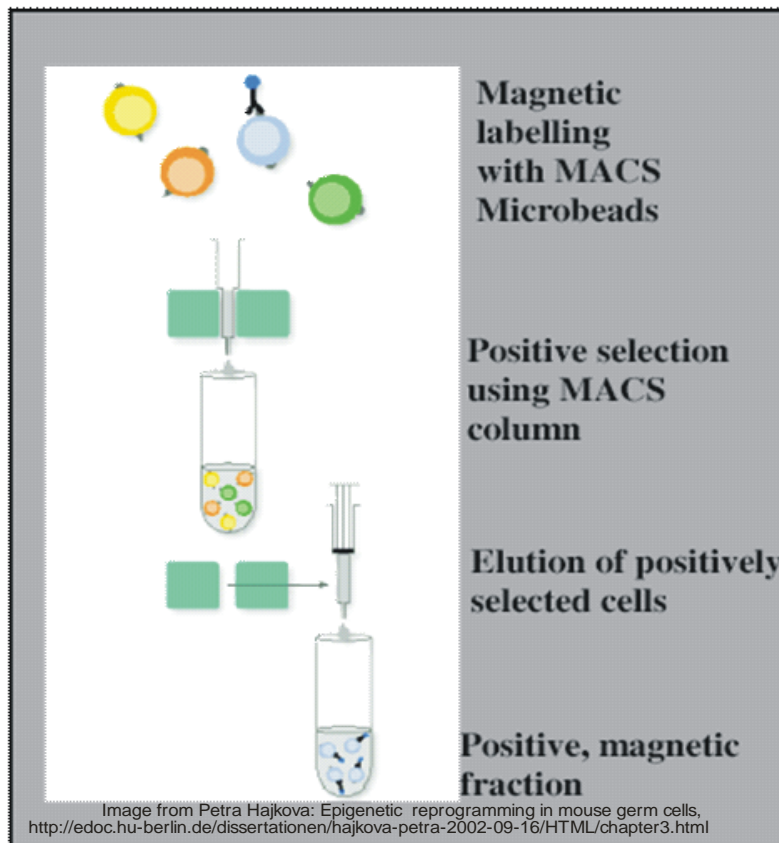


Figure 3: MACS Method

2.5 Flow cytometric analysis

After magnetic cell sorting, a fraction of the positively sorted cells were characterized by flow cytometry using CD14, CD34, CD45, CD73, CD90, and CD105 antibodies (all from Becton Dickinson, Heidelberg, Germany), CD146/MUC18 (eBioscience, NatuTec GmbH) and STRO-1 (BioLegend). Binding of the primary antibodies and the corresponding isotype controls was performed according to standard protocols by using r FcR Blocking Reagent

(Miltenyi Biotec) and evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson). Curves were plotted for markers on cells.

2.6 Cryopreservation of the cells

Samples of cultures obtained before as well as after the magnetic cell sorting from the two described protocols were preserved in liquid nitrogen.

The cells from cultures desired for preservation were detached, transferred to a new Falcon tube and centrifuged for 5 min at 1500 rpm. Afterwards, the pellet was resuspended in 1 ml freezing medium consisting of Minimum Essential Medium Eagle Alpha Modification and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich) and transferred to cryo-tubes. The cells were immediately preserved at -70°C. After a few days the cells were transferred into liquid nitrogen for final preservation.

2.7 Colony-forming unit (CFU) assay

To assess colony-forming efficiency, cells were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% amphotericine medium at a density of 5 cells/cm² in 20 cm² culture wells.

At day twelve cultures were fixed with 4% formalin and stained with 0.1% crystal violet (Sigma-Aldrich). Aggregates of 50 or more cells were scored as colonies.

2.8 Osteogenic, adipogenic and chondrogenic differentiation

Cells obtained by the two different isolation protocols were independently induced to differentiate into osteogenic, adipogenic and chondrogenic lineages. Cells at the fourth passage were cultured on 6-well culture plates for osteogenic and adipogenic and in 1.5 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) for chondrogenic differentiation with specific inductive media (figure 4).

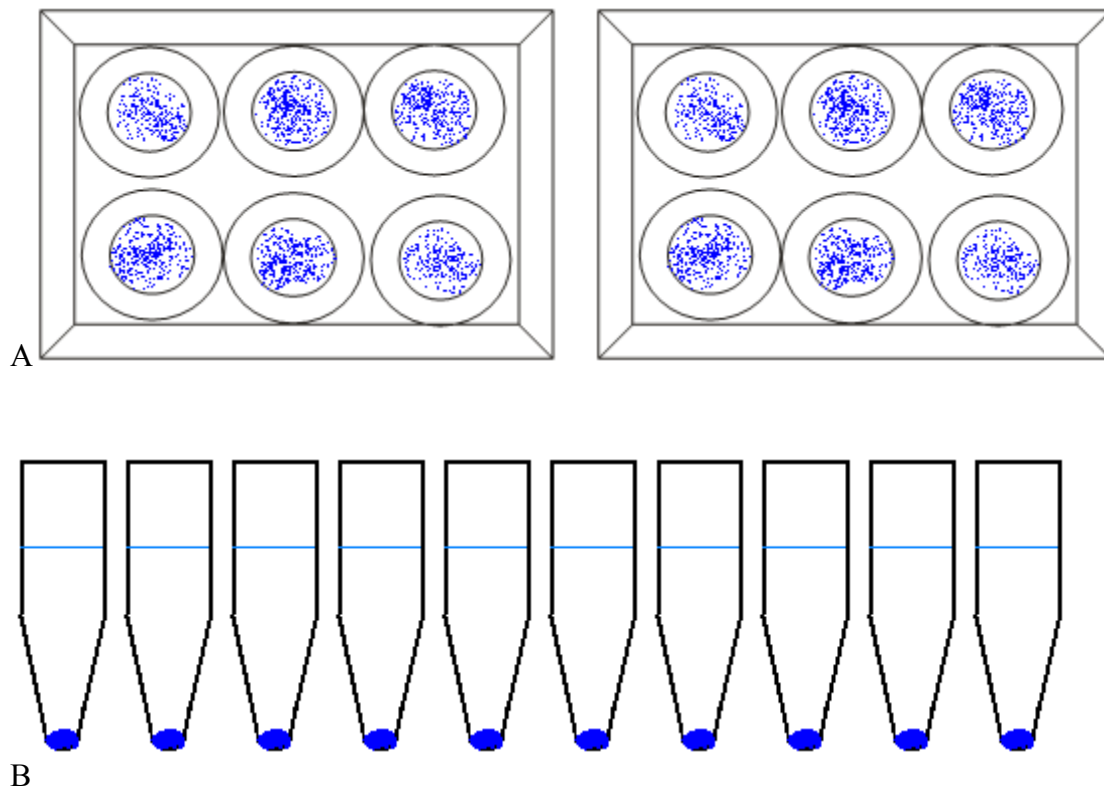


Figure 4: Schematic drawing of (A) 6-well culture plates for osteogenic and adipogenic differentiation and (B) 1.5 ml Eppendorf tubes for chondrogenic differentiation of the isolated cells (blue masses).

2.8.1 Osteogenic differentiation

To test for osteogenic differentiation potential, cells were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 1% amphotericine at a density of 2×10^4 cells per well on 6-well culture plates. Upon reaching confluence, cells were cultured in media consisting of Minimum Essential Medium Eagle Alpha Modification, 15% FCS, 100 $\mu\text{g/ml}$ streptomycin and 1% amphotericine, dexamethasone (0.1 μM) (Sigma-Aldrich), β -glycerophosphate (10 mM) (Sigma-Aldrich), ascorbic acid (50 $\mu\text{g/ml}$) (Sigma-Aldrich). As a control cells from the two protocols were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 1% amphotericine. The media were renewed three times a week. To assess the differentiation potential into osteogenic lineage, at day 14 cultures were stained with Alizarin Red (Sigma-Aldrich) to distinguish the presence of calcified deposits.

2.8.2 Adipogenic differentiation

To test for adipogenic differentiation potential, cells were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine at a density of 3×10^5 cells per well on 6-wells culture plates. Upon reaching confluence, cells were cultured in media consisting of Minimum Essential Medium Eagle Alpha Modification, 15% FBS, 100 µg/ml streptomycin and 1% amphotericine, dexamethasone (1 µM), insulin (10 µg/ml) (Sigma-Aldrich), 1-methyl-3-isobutylxanthin (100 µg/ml) (Sigma-Aldrich), indomethacin (60 µM) (Sigma-Aldrich) and L-glutamine (4 mM). As a control, cells from the two protocols were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine. The media were renewed three times a week.

The presence of lipid droplets was evaluated in the cultures at day 21 through staining with Oil Red O to determine the differentiation into adipogenic lineage cells. Also the cells were examined for the expression of the marker lipoprotein lipase (LPL) on mRNA level.

2.8.3 Chondrogenic differentiation

To test for chondrogenic differentiation potential, cells at a density of 30,000 cells per tube were cultured in 1.5 ml Eppendorf tubes. This was done with micro-masses that were incubated for one hour before adding the chondrogenic inductive medium (PromoCell, Heidelberg, Germany). As a control, cells from the two protocols were cultured in Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericine. The media were renewed three times a week.

Chondrogenic differentiation was evaluated at day 35 through staining glycosaminoglycans present in the cultures by the application of Alcian Blue (Sigma- Aldrich).

2.9 Gene expression profile

2.9.1. RNA extraction and purification

RNA extraction was done using the RNeasy kit (Qiagen, Hilden, Germany). Medium was aspirated off the wells and the cells were washed twice with PBS. Afterwards, cells were scraped in PBS and disrupted by addition of buffer RLT with β-mercaptoethanol. The sample

was homogenized and 1 volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. 700 μ l of the sample were applied to an RNeasy mini column placed in a 2 ml collection tube. The tube was closed gently, centrifuged for 30 s at 10,000 rpm and the flow-through discarded. 700 μ l Buffer RW1 was added to the RNeasy column, the tube closed gently, centrifuged for 30 s at 10,000 rpm and the flowthrough discarded. The RNeasy column was transferred into a new 2 ml collection tube and 500 μ l Buffer RPE pipetted onto the RNeasy column. The tube was closed gently, centrifuged for 30 s at 10,000 rpm and the flowthrough discarded. Finally, the RNeasy column was transferred to a new 1.5 ml collection tube, 30–50 μ l RNase-free water directly pipette onto the RNeasy silica-gel membrane. The tube was closed gently, centrifuged for 1 min at 10,000 rpm.

The RNA obtained was purified using RNase-free DNase I (Promega, Mannheim, Germany), quantified photometrically and the required RNA dilutions for 8 μ l solution calculated.

2.9.2 Reverse transcription

The reverse transcription steps were performed using the SuperScript reverse transcription kit (Qiagen). On ice 8 μ L mRNA were pipette per tube together with 1 μ L oligo dT (20 μ M) and 1 μ L dNTP mix (10 μ M each of dATP, dCTP, dGTP, and dTTP)

Fresh master-mix was prepared on ice as:

- 10x Buffer RT
- MgCl₂ 25 mM
- DTT 0.1 M
- RNase out
- SS II RT (Superscript)

Tubes were put into the thermal cycler (Mastercycler gradient, Eppendorf) and after a 2-min denaturation step 10 μ L master-mix were added per tube while in the cycler. The cycler program was run for an additional 50 min and the SuperScript reverse transcription kit heat inactivated for 15 min. Subsequently, 1 μ l RNase H (Qiagen) was added per tube and the cycler continued for an additional time of 20 min until the cDNA reaction was terminated.

2.9.3. Real-time Polymerase Chain Reaction (real time-PCR)

Using a real time-PCR cycler (LightCycler, Roche Molecular Biochemicals, Indianapolis, Indiana, USA) polymerase chain reaction (PCR) was performed. Briefly, for each sample 160 ng of cDNA template was added to 10 μ L of QuantiTect SYBRgreen master mix (Qiagen)

and 1 μ l of both forward and reverse primer (10 μ M each final concentration) up to a total volume of 20 μ l with RNA-free water. PCR results were quantified by using standard curves generated from external standard templates. The cDNA for external standards was generated by nested RT-PCR from total RNA obtained from the cell. Copies per ml were calculated according to the following formula:

$$\frac{\text{Molecules}}{\mu\text{l}} = \frac{\text{DNA concentration } (\mu\text{g/ml}) \times 6.022 \times 10^{23}}{(660 \times 10^9 \times \text{product length})}$$

Copy numbers from 1×10^2 to 1×10^{11} were submitted to real time-PCR to generate standard curves. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was run in parallel with all experimental samples.

Table 1: Primer sequences used in the PCR

Gene	GenBank accession number	Forward Primer	Reverse Primer	Product size
Alkaline Phosphatase (long)	NM_000478.4	ccacgtcttcacatttggtg	agactgcgccttgtagttgt	196
Alkaline Phosphatase (short)	NM_000478.4	cccgtggcaactctatcttt	catggagacattctctcgttca	131
Osteopontin (long)	J04765.1	cccacagaccctccaagta	acactatcacctcgccatc	279
Osteopontin (short)	J04765.1	acagccaggactccattgac	ggggacaactggagtgaaaa	161
Osteonectin (long)	M25746.1	ctctttaaccctccccttcg	atgggcaaagctacaaatgg	230
Osteonectin (short)	M25746.1	tggatggtttgttctctgc	gggactattaatgcgtgtgga	153
Osteocalcin (long)	M34013.1	ccccgcttctcttagact	agggtgagccacaatcagac	163
Osteocalcin (short)	M34013.1	cccctagagctcagccagt	gctcttgacactgctctgg	117
Collagen I (long)	NM_000089.3	ctgcaagaacagcattgcat	ggcgtgatggcttatttgtt	203
Collagen I (short)	NM_000089.3	atgaggagactggcaacctg	caatgattgtctttcccatt	150
Collagen III (long)	NM_000090.3	tacggcaatcctgaactcc	gtgtgttctgcaaccatc	245
Collagen III (short)	NM_000090.3	catctggcattcctcgact	tgctatttccctcagcctga	163
Collagen V (long)	BC008760.2	ggctgtgctaccaagaaagg	gaggtcacgaggttctctc	198
Collagen V (short)	BC008760.2	acggttctggagatcgacac	gccccacttcaaatccaat	106
GAPDH (long)	NM_002046	cctgacctgccgtctagaaa	tactccttgaggccatgtg	276

GAPDH (short)	NM_002046	tcaagaagtggtgaagcag	ccctgtgctgtagcctcaat	198
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Cycling conditions were set to 95°C for 15 min (initial denaturation) followed by 45 cycles of 94°C for 15 s (denaturation), 56°C for 30 s (annealing), and 72°C for 75 s (extension). The number of copies was extrapolated from the corresponding standard curve and expressed relative to 1 µg of RNA introduced into the RT reaction and normalized to GAPDH. PCR products were separated and analyzed to control for single amplification products and for the expected product size by agarose gel (3%) electrophoresis. Specificity of amplification reaction was further controlled by melting curves, no template controls, and no RT controls.

3. Results

Both described protocols showed significant results regarding the selected criteria for the alleged periodontal stem cells isolation and characterization.

3.1 Phase contrast inverted microscopy

One week after the initial adherence phase of the alveolar bone and gingival cervical soft tissue parts according to the two described methods, cells from both protocols began to grow out of the adherent tissue masses. Tissue cultures were periodically checked every 24 hours by the phase contrast inverted microscopy. The outgrowing cells showed a fibroblastic appearance (figures 5 & 6).

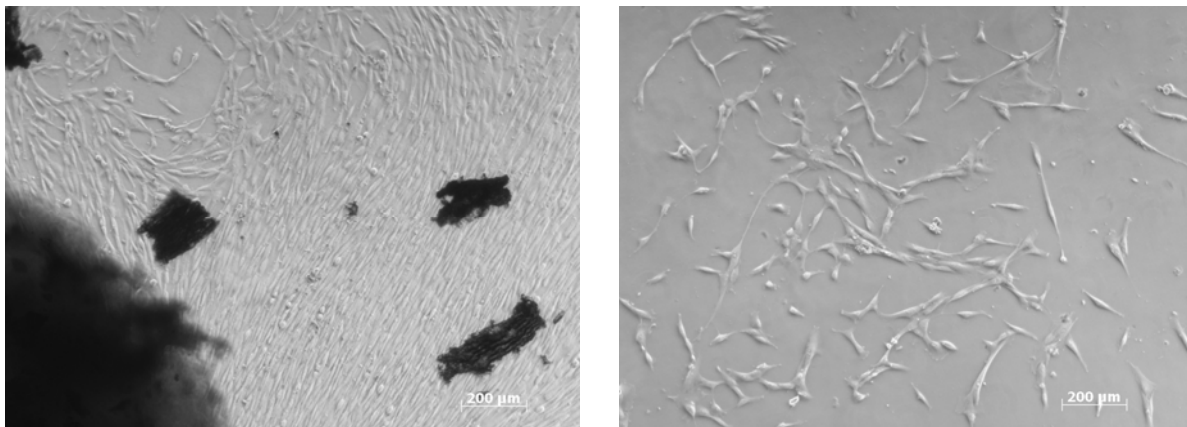


Figure 5: Microscopic analysis of cells derived from the alveolar bone after 1 week and after first cell passage

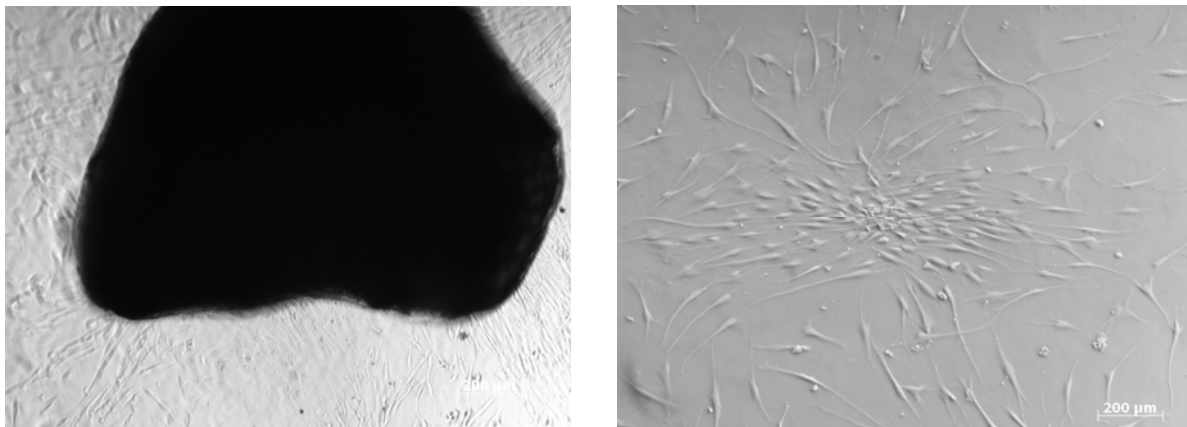


Figure 6: Microscopic analysis of cells derived from the gingival margin after 1 week and after first cell passage

3.2 Flow cytometric analysis

On performing the flow cytometric analysis following the magnetic cell sorting, cells derived from the two protocols were negative for the cell surface markers CD14, CD34, and CD45 (<2%). Regarding the cell surface markers CD73, CD90, CD105, CD146, and STRO-1, cells from the two protocols showed positive signals. The percentage of positive cells varied between the two protocols, particularly for the markers CD146 and STRO-1 (figures 7 & 8).

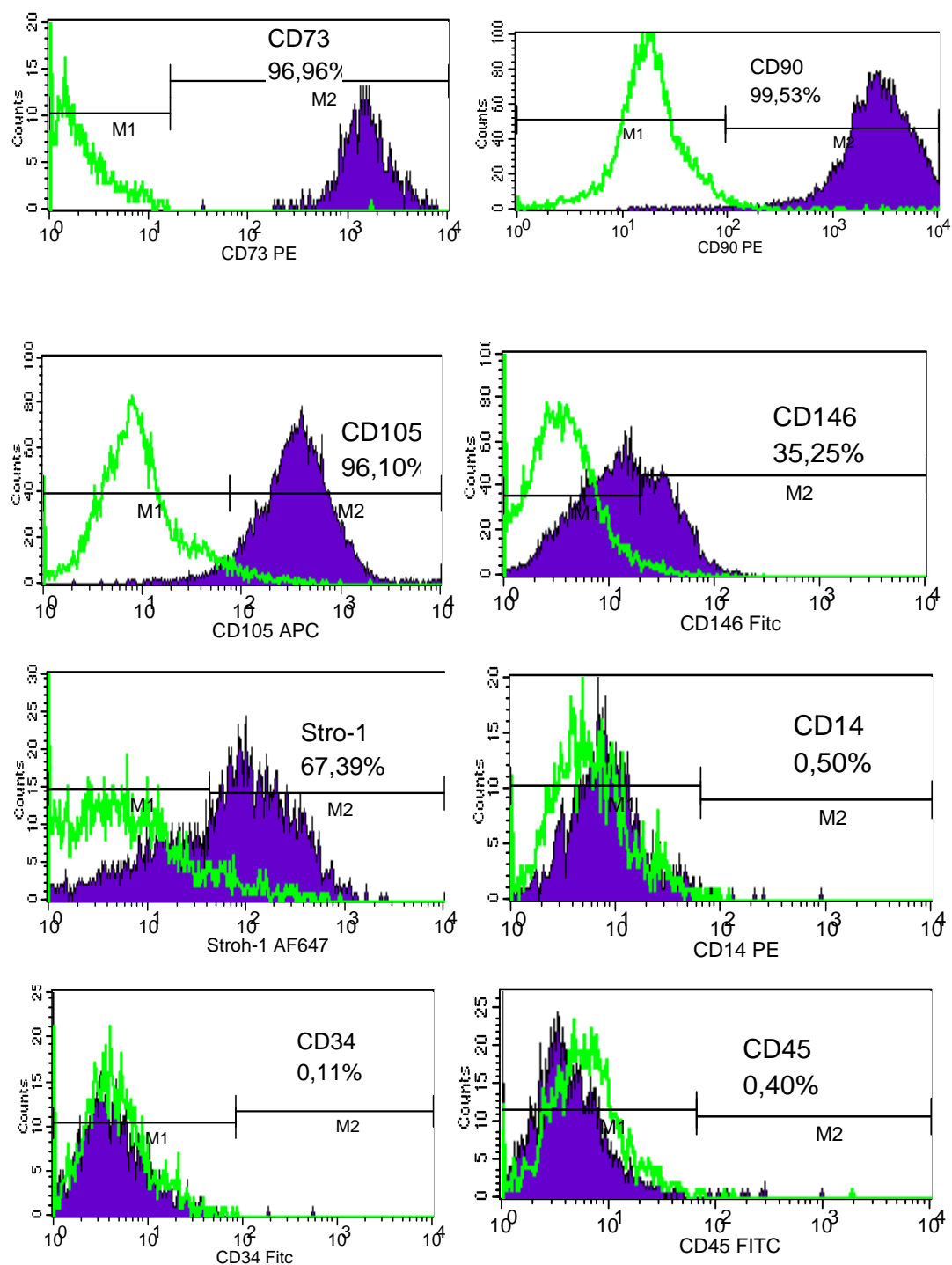


Figure 7: Surface markers' expression on cells derived from the alveolar bone

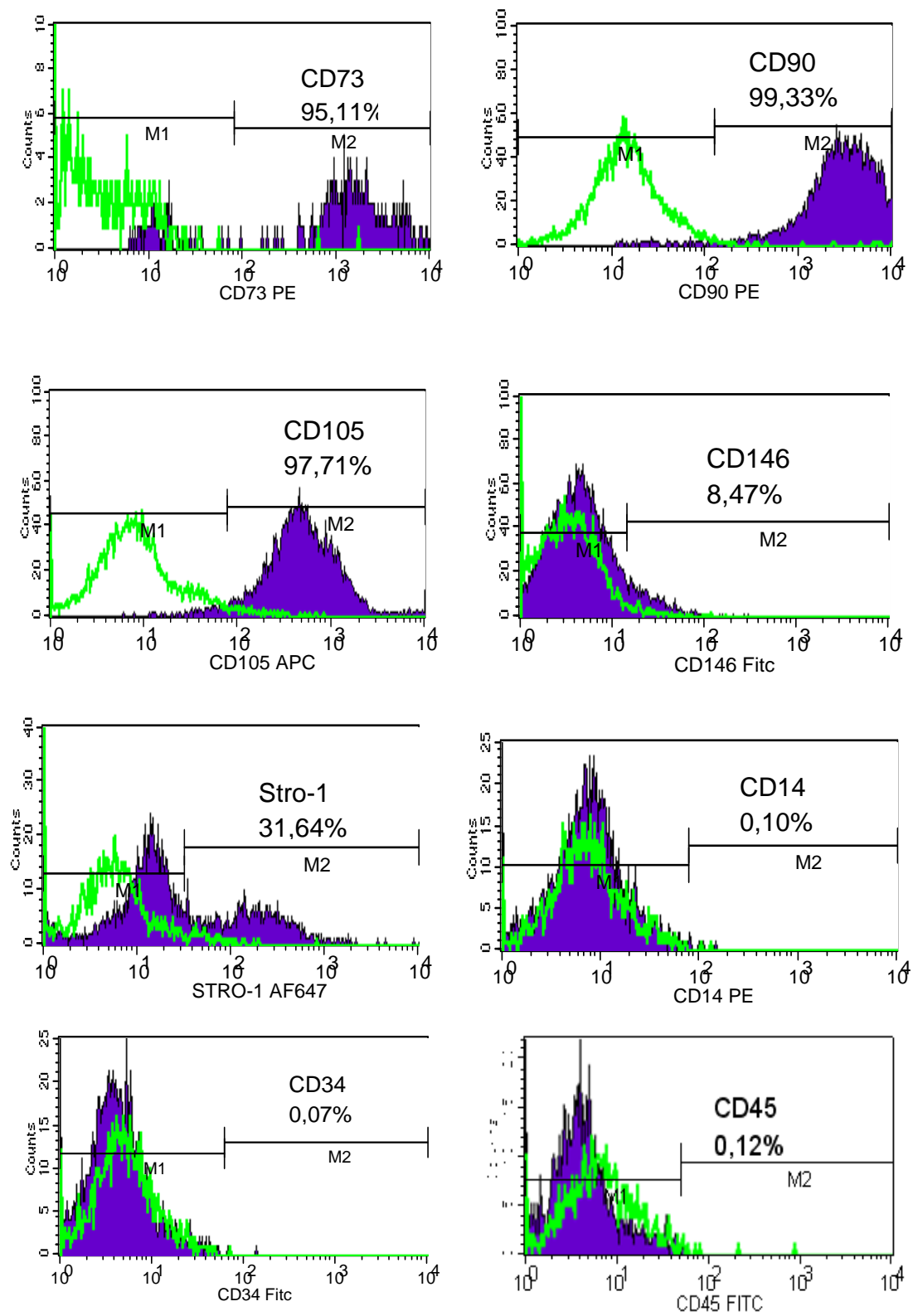


Figure 8: Surface marker expression of cells derived from the gingival cervical margin

3.3 Colony forming unit (CFU) assay

Twelve days after seeding cells prepared according to both protocols showed colony-forming units (CFUs) (figures 9 & 10). In contrast to periodontal fibroblasts seeded under identical culture conditions, failed to demonstrate the same property, but showed a more diffuse distribution pattern (figure 11). The adherent clonogenic cell clusters of fibroblast-like cells resembled those of other mesenchymal stem cell populations.

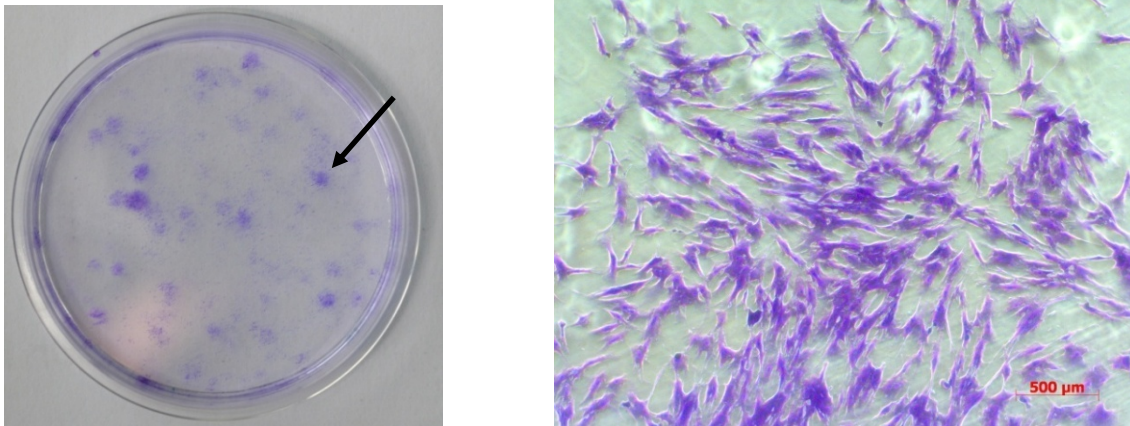


Figure 9: Colony forming unit assay in cells derived from the alveolar bone

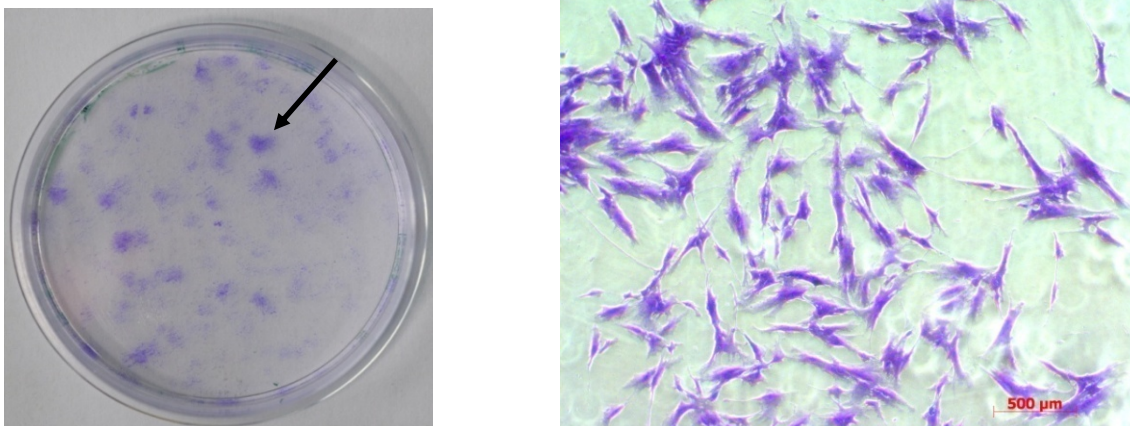


Figure 10: Colony forming unit assay in cells derived from the gingival cervical connective tissue

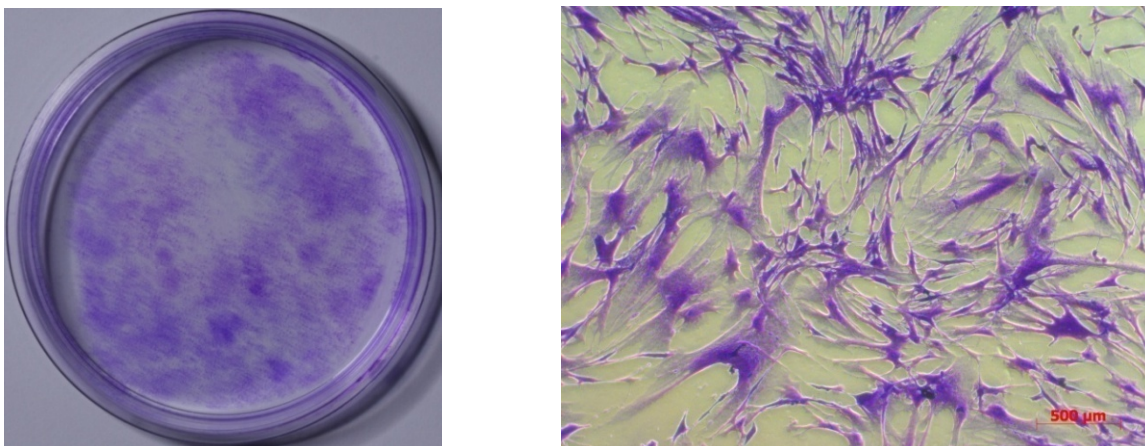


Figure 11: Colony forming unit assay in periodontal fibroblasts

3.4 Osteogenic differentiation

Cells differentiated according to the two protocols were cultured on 6-well culture plates for two weeks in an osteogenic inductive medium. At the end of this process, by staining with Alizarin Red, the presence of calcium deposits was observed, indicating osteogenic differentiation of the isolated cells from the alveolar bone as well as the gingival margin. In contrast, the control cells lacked Alizarin Red-positive deposits (figures 12 & 13).

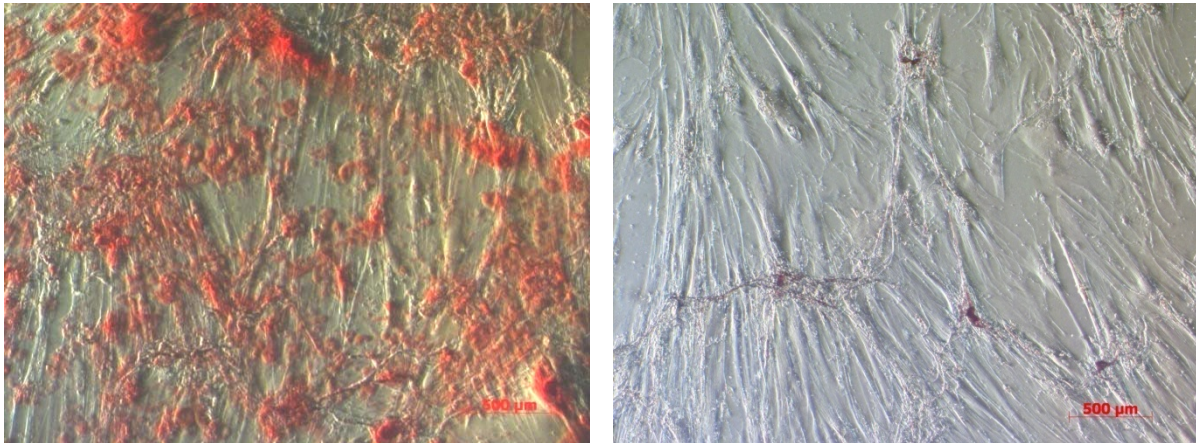


Figure 12: Osteogenic differentiation as revealed by Alizarin Red staining of alveolar bone derived stem cells with osteogenic inductive (left) and control (right) media

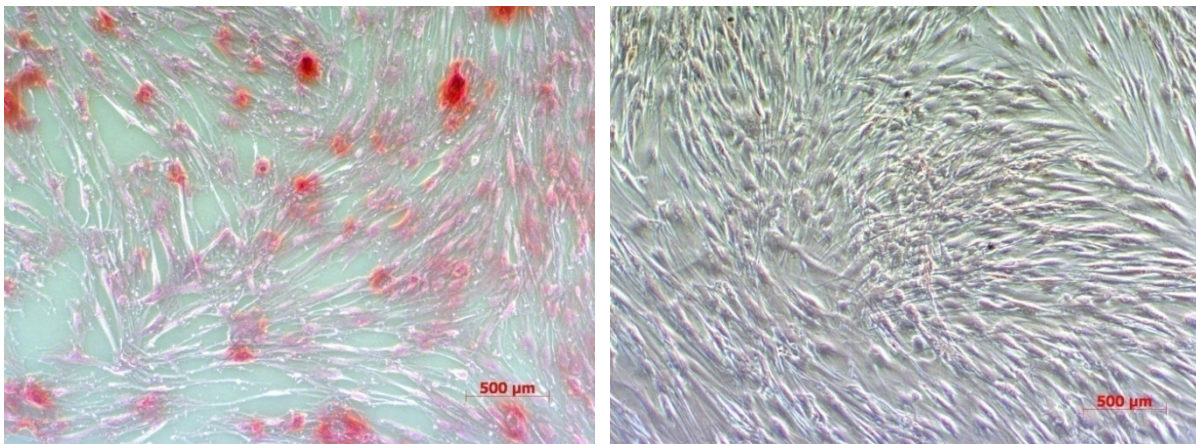


Figure 13: Osteogenic differentiation as revealed by Alizarin Red staining of gingival margin derived stem cells with osteogenic inductive (left) and control (right) media

3.5 Adipogenic differentiation

Cells prepared according to both protocols were cultured on 6-well culture plates for three weeks in an adipogenic inductive medium. At the end of this process, by staining with Oil Red O, the presence of lipid droplets was observed, indicating adipogenic differentiation of the isolated cells from the alveolar bone as well as the gingival margin, in contrast to controls which lacked Oil Red O staining (figures 14 & 15).

On the gene level the adipogenically differentiated cells from both protocols expressed LPL as evidenced by PCR (3% agarose gel electrophoresis) in contrast to the control cells, which appeared negative for LPL (figures 16 & 17).

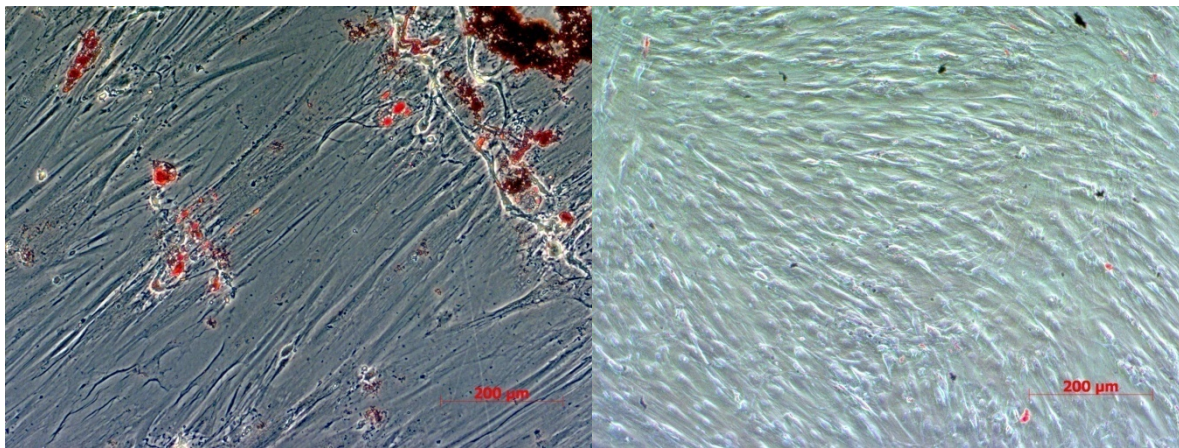


Figure 14: Adipogenic differentiation as revealed by Oil-Red-O staining of alveolar bone derived stem cells with adipogenic inductive (left) and control (right) media

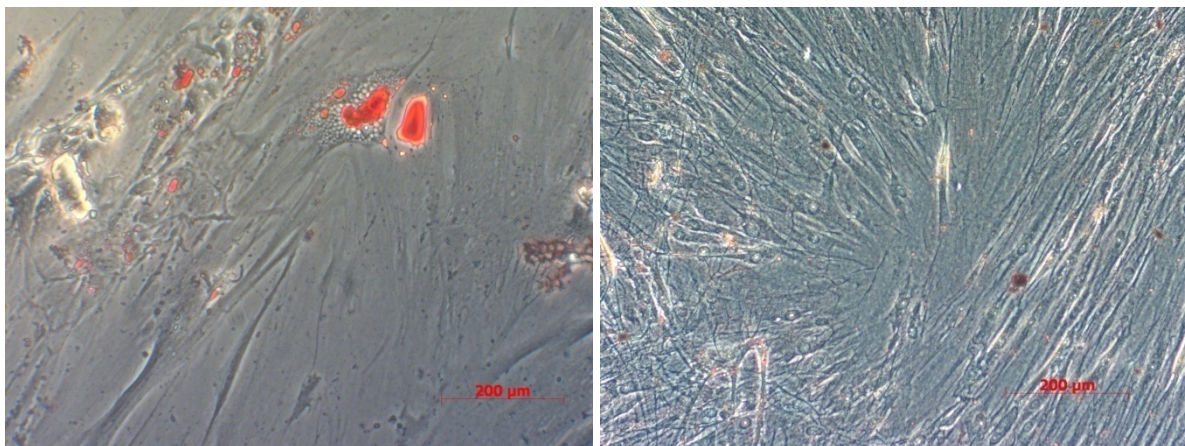


Figure 15: Adipogenic differentiation as revealed by Oil-Red-O staining of gingival margin derived stem cells with adipogenic inductive (left) and control (right) media

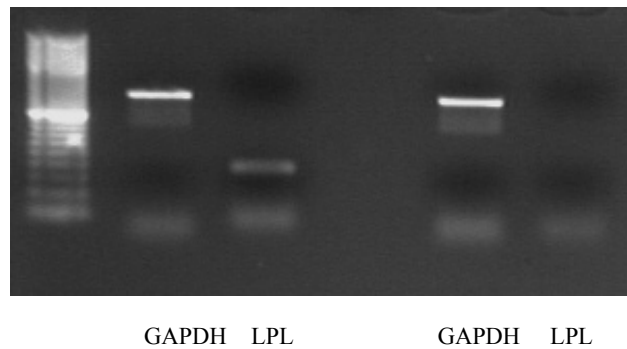


Figure 16: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for GAPDH and LPL expression in alveolar bone derived cells with adipogenic inductive (left) and control (right) media

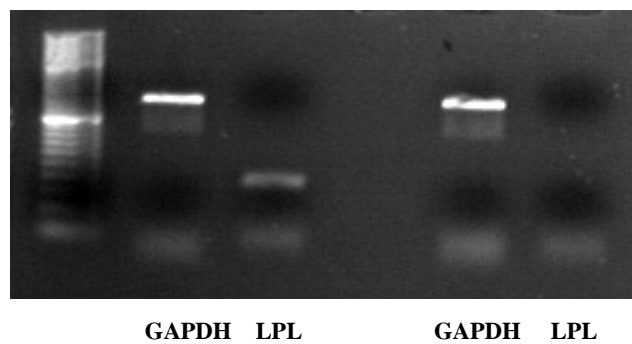


Figure 17: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for GAPDH and LPL expression in gingival margin derived cells with adipogenic inductive (left) and control (right) media

3.6 Chondrogenic differentiation

Cells generated according to the two protocols were cultured in 1.5 ml Eppendorf tubes for five weeks in a chondrogenic inductive medium. At the end of this process, by staining with Alcian Blue, the presence of glycosaminoglycans was observed, indicating chondrogenic differentiation of the isolated cells from the alveolar bone as well as the gingival margin, in contrast to the respective controls lacking Alcian Blue staining (figures 18 & 19).

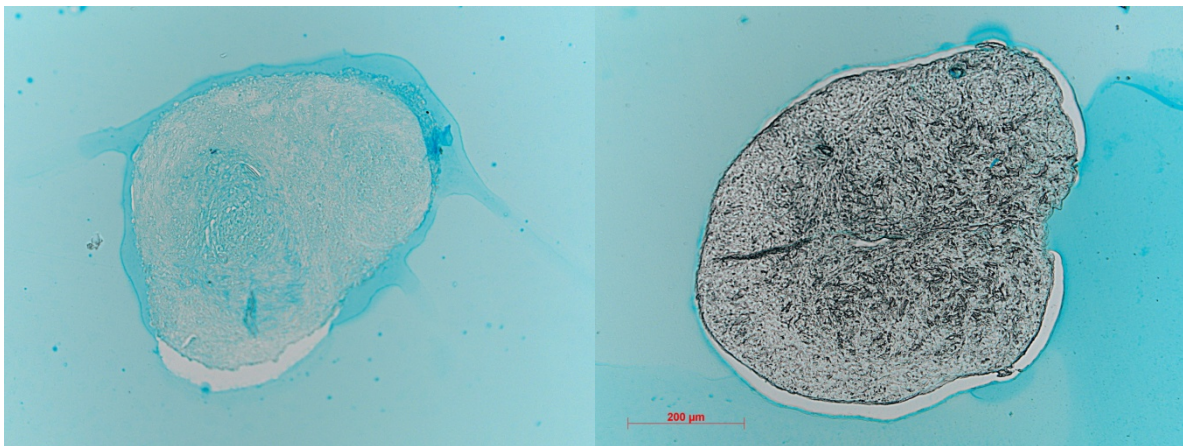


Figure 18: Chondrogenic differentiation as evidenced by Alcian Blue staining of alveolar bone derived stem cells with chondrogenic inductive (left) and control (right) media

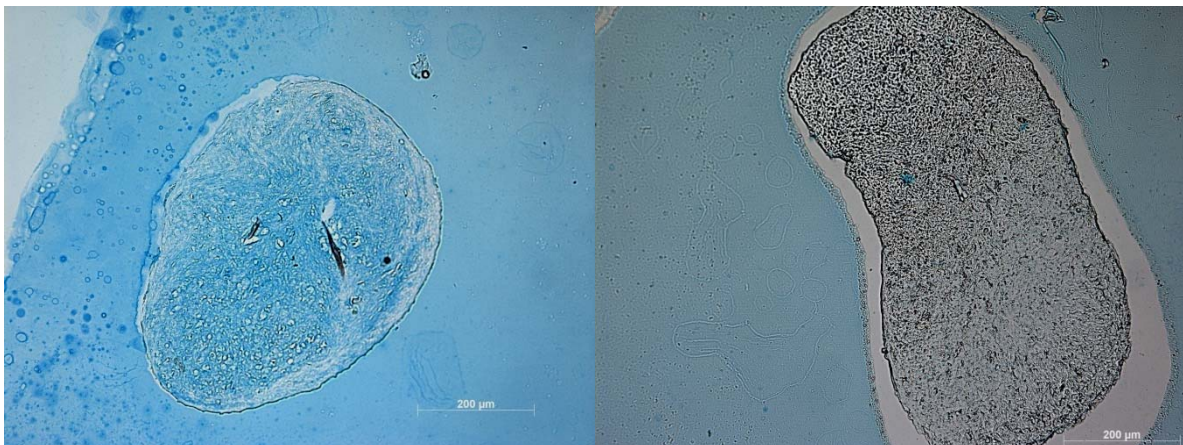


Figure 19: Chondrogenic differentiation as evidenced Alcian Blue staining of gingival margin derived stem cells with chondrogenic inductive (left) and control (right) media

3.7 Gene expression profile

On the gene level, unstimulated cells derived from both the alveolar bone and gingival cervical margin showed positive expression for mRNA of all tested osteogenic markers.

The expression varied between unstimulated cells from the gingival cervical margin, from the alveolar bone as well as periodontal fibroblasts (figures 20 & 21). Although the intensity of the expression varied between the cells derived from the gingival cervical margin as well as from the alveolar bone, they were qualitatively similar with respect to fragment size (figures 22 & 23).

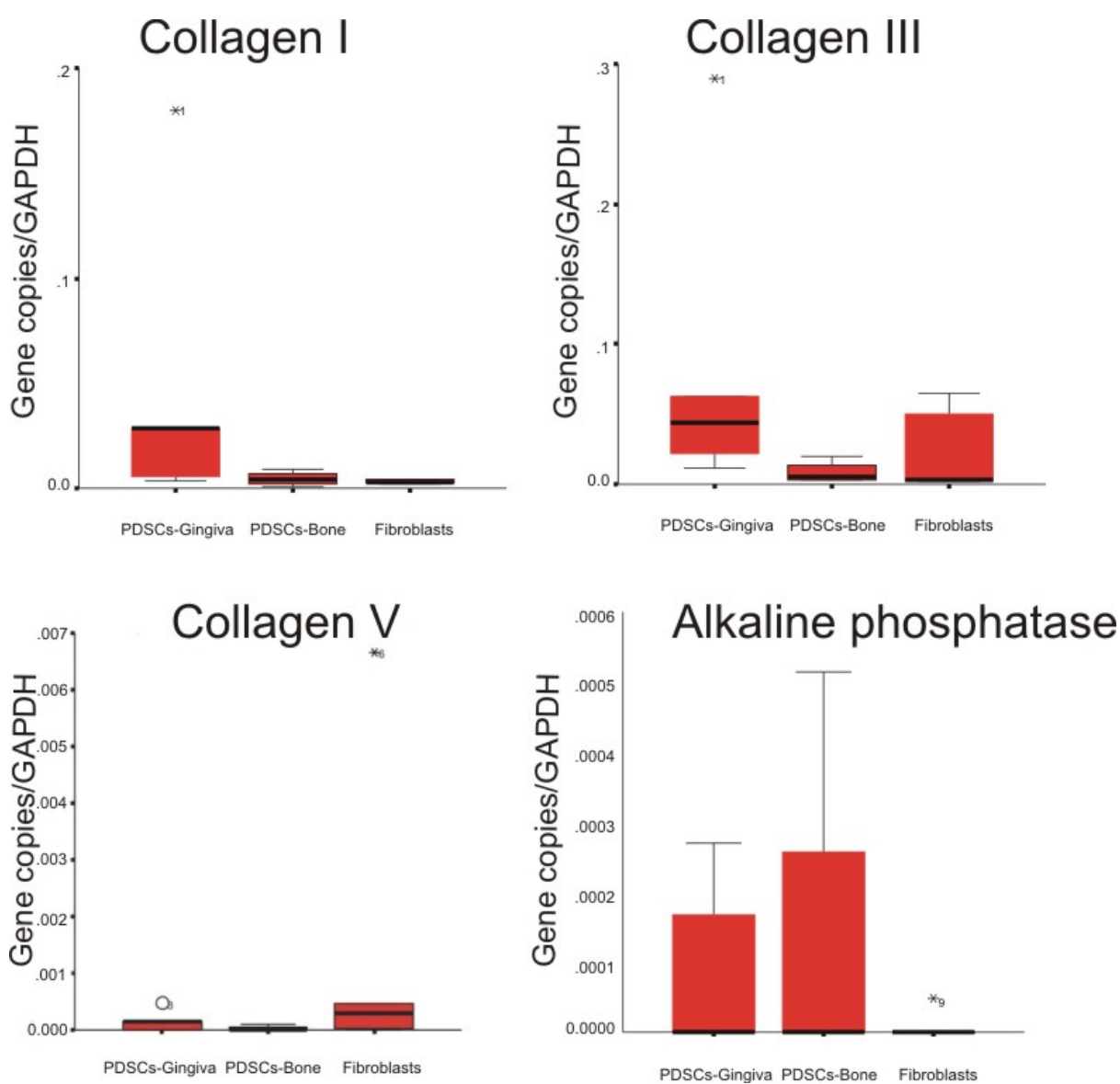


Figure 20: Gene expression profile of unstimulated cells derived from the gingival margin (PDSCs-Gingiva), alveolar bone (PDSCs-Bone) as well as periodontal fibroblasts (Fibroblasts) for Collagen I, III and V and alkaline phosphatase as determined by real-time PCR (boxplots).

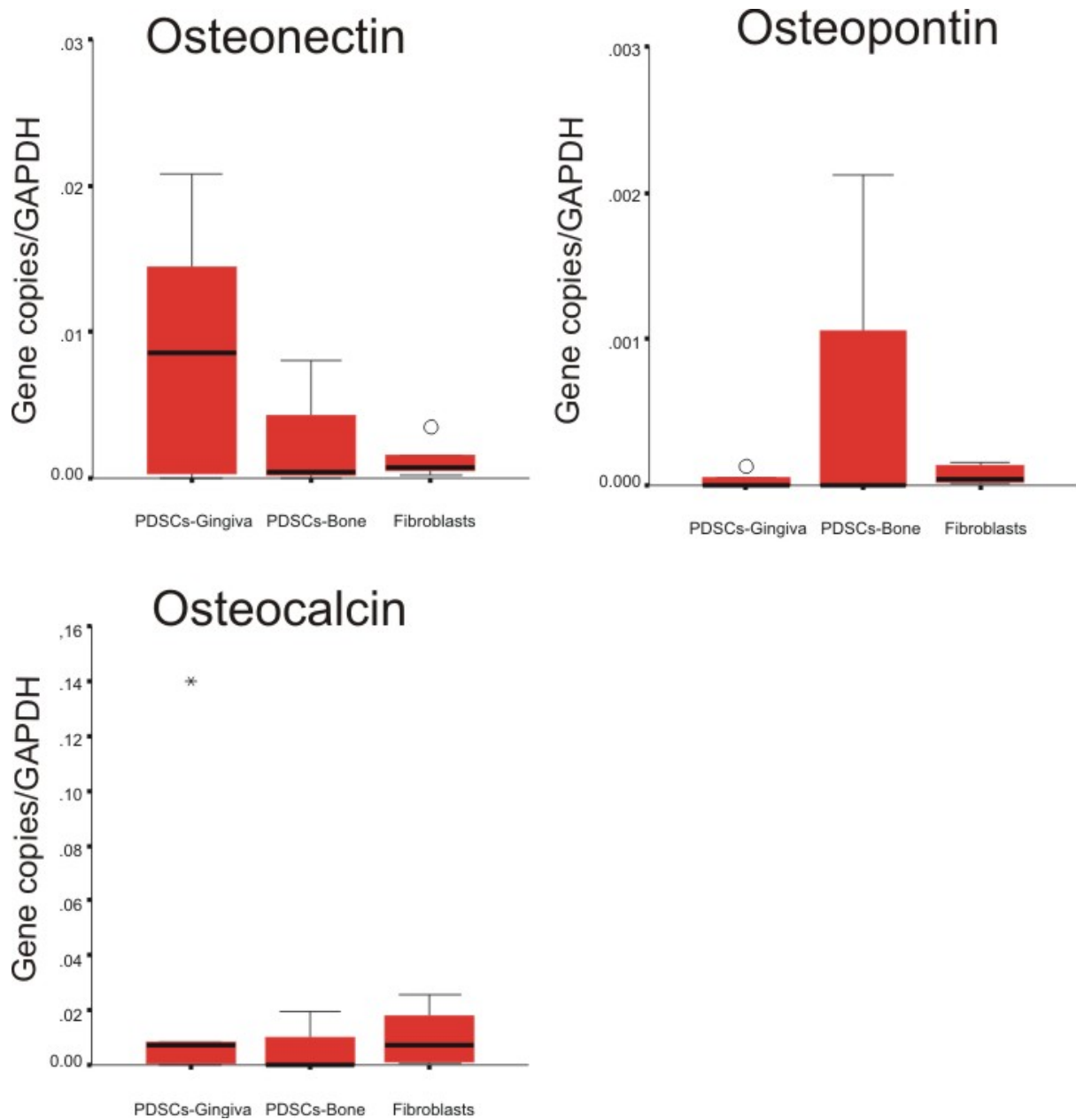


Figure 21: Gene expression profile of unstimulated cells derived from the gingival margin (PDSCs-Gingiva), alveolar bone (PDSCs-Bone) as well as periodontal fibroblasts (Fibroblasts) for osteonectin, osteopontin and osteocalcin as determined by real-time PCR (boxplots).

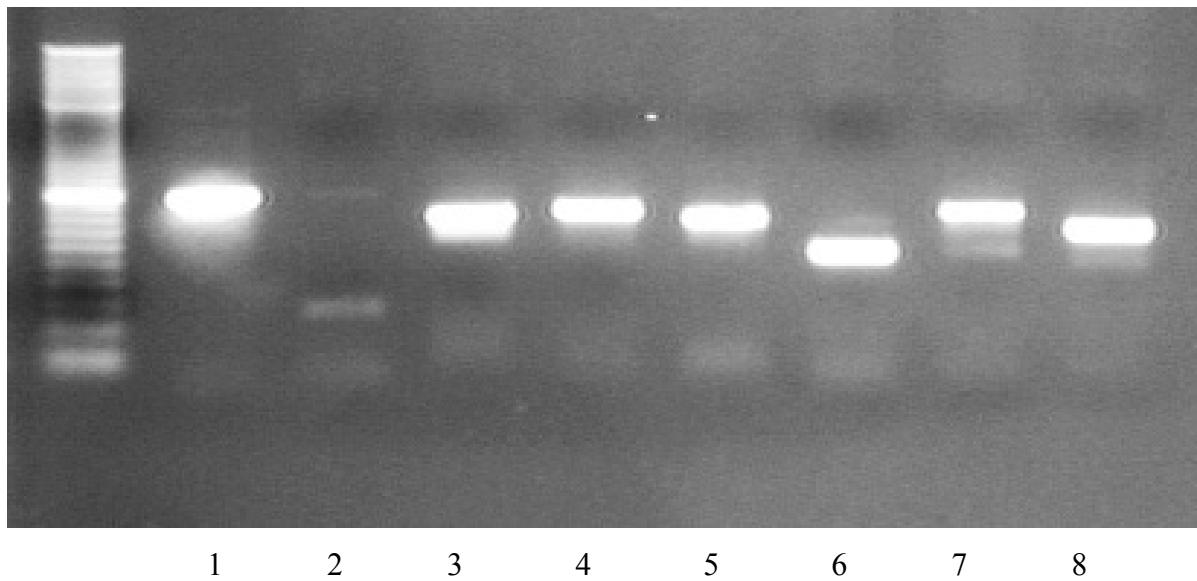


Figure 22: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for (1) GAPDH, (2) Osteocalcin, (3) Osteonectin, (4) Osteopontin, (5) Collagen I, (6) Collagen V, (7) Collagen III and (8) ALP expression in unstimulated cells derived from the alveolar bone.

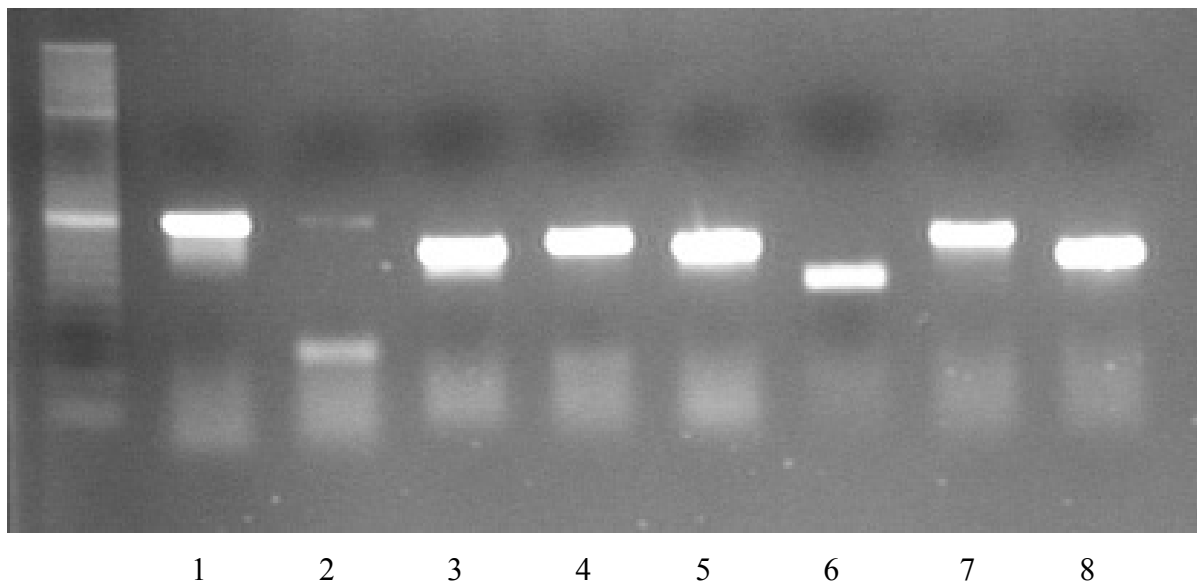


Figure 23: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for (1) GAPDH, (2) Osteocalcin, (3) Osteonectin, (4) Osteopontin, (5) Collagen I, (6) Collagen V, (7) Collagen III and (8) ALP expression in unstimulated cells derived from the gingival margin.

4. Discussion

4.1 Methods

4.1.1 Identification criteria

The aim of the present study was to isolate as well as to characterise adult multipotent periodontal stem cells (PDSCs) from both the alveolar bone and the gingival cervical margin of the periodontium. To tackle this issue, the standards accepted by the International Society for Cellular Therapy in their position paper (Dominici et al. 2006) to define human MSCs (or as they refer to them: multipotent stromal cells) as well as the criteria for identifying periodontal stem cells as defined by Bartold et al. (2006) in their review article were combined and the study design deduced from them. They were taken as guidelines and the design and execution of the current study strictly adhered to them.

The combined criteria for the identification of the stem cells in this study included, firstly, that the alleged MSCs are plastic-adherent when maintained under standard culture conditions, secondly, that more than 95% of the alleged MSCs' population express, as measured by flow cytometry, the surface markers CD73, CD90, and CD105 (with further expression of CD146 and STRO-1 and lack of expression (less than 2%) of CD45, CD34, CD14), and thirdly, that the cells show colony-forming ability. Fourthly, these cells must be able to differentiate into osteoblastic, adipocytic and chondroblastic directions under standard in vitro differentiating conditions. Finally, these unstimulated stem cells should ideally express mineralized tissue markers on the mRNA level.

4.1.2 Sample preparation

For culturing PDSCs from the isolated alveolar bone as well as the gingival cervical soft tissue parts standard culture protocols were employed. The isolated tissues were not enzymatically digested and were not exposed to extensive mechanical manipulation. This approach was different to the one described by Seo et al. (2004) to isolate PDLSCs. In their study, tissues were first scraped from the root surface and digested with collagenase I (to digest any remaining collagen fibrils) and dispase (to digest cellular attachment plaques). The resulting cellular suspensions were passed through cell strainers and were seeded in tissue culture dishes. In the current study we omitted enzymatic digestions and extensive mechanical manipulation, as we believe that this would convey extra stress on the cells or

even harm them. The idea was further to simplify the tissue manipulation procedures as much as possible, thereby developing readily applicable culture techniques.

However, the plastic adherence of the isolated cells, which allowed for the first identification of any potential mesenchymal stem cell, was of utmost importance. Cells were left to adhere to the unmodified surface of tissue culture flasks and allowed to grow out of their alveolar bone as well as gingival margin tissue parts. Only adherent cells were accepted and any floating cell or tissue parts were discarded.

4.1.3 Magnetic activated cell sorting (MACS)

During the last 30 years the use of magnetic particles has been well established as a standard tool for the isolation of defined cell subsets in modern cell biology and immunology. In 1977, Molday et al. published their work on the use of iron-containing polymeric microspheres conjugated to lectins for the separation of red blood cells in addition to antibody-coated cells. Since then magnetic cell sorting has become a standard and well established cell isolation technique.

The biological and optical inertness of nanoparticles ranging from 20 to 100 nm in diameter is the main reason why colloidal super-paramagnetic particles (MACS-beads) have become the gold standard for magnetic cell separation over the last 20 years. High-gradient magnetic cell separation columns are used for magnetization of labelled cells in a magnetic field generated by a strong external magnet. These columns are filled with a matrix of ferromagnetic steel-wool or iron-spheres, which focus the magnetic field lines towards their surface and so induce strong magnetic field gradients (4 Tesla), which attract even slightly magnetic cells and absorb them to their surface. Outside the magnet, the ferromagnetic matrix can no longer retain the labelled cells and they can be eluted. Magnetic cell sorting has evolved from the original fluorescence activated cell sorting (FACS)-support technology to a real alternative for many applications. It is fast, provides comparable purity and efficacy, and is less stressing for the cells.

For magnetically isolating the potential PDSCs from both the alveolar bone and gingival soft tissue, STRO-1 antibodies together with anti-IgM MicroBead antibodies were utilized, similar to standard isolation protocols employed for the isolation of BMSSCs from bone marrow aspirate (Gronthos et al. 2008). The principle relies on the fact that the STRO-1 antibodies together with anti-IgM MicroBead antibodies attach to the desired cells and subsequently become attracted to the magnetic field. Outside the magnet, the ferromagnetic matrix was unable to retain the labelled cells and they were subsequently eluted. This

technique thereby succeeded in enriching cells expressing STRO-1 from both sources and from mixed cultures. However, although this technique has many advantages including simplicity, rapidness, and low stress for the cells, it is not suitable to enrich the cells to 100% from mixed cultures.

4.1.4 Flow cytometric analysis

Flow cytometry is a technique for counting and examining microscopic particles, such as cells surface markers. The technique is based on the idea that the examined cells coated with fluorescent antibodies are suspended in a fluid. The stream of fluid with the cells is subsequently passed through an electronic detection apparatus. A beam of light of a single wavelength is directed onto the hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. Each suspended particle ranging from 0.2 to 150 μm in size and passing through the beam scatters the ray and fluorescent chemicals found in or attached to the particle becomes excited and, as a consequence, emits light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector, it is subsequently possible to derive various types of information about the physical and chemical structure of each particle.

This highly accurate technique was used in this study to characterize the surface marker expression of the PDSCs derived from both the alveolar bone as well as from the gingival cervical margin. The selected surface markers were CD14, CD34, CD45, CD73, CD90, CD105, CD146, and STRO-1. As mentioned above, this specific marker constellation was based on the work of Dominici et al. (2006) and Bartold et al. (2006). However, each of these markers has a characteristic cell surface expression profile, defining its importance as an identification criterion for multipotent mesenchymal stem cells.

CD14 is normally a component of the innate immune system and should therefore not be expressed on MSCs. It is expressed mainly on peripheral blood monocytes, macrophages, neutrophil granulocytes, and dendritic cells. It primarily functions as a co-receptor for bacterial lipopolysaccharide (LPS) (Thomas et al. 2002).

CD34 is a member of a family of transmembrane sialomucin proteins that are expressed on early hematopoietic and vascular-associated tissue. It functions as an important adhesion molecule and is required for T cells to enter lymph nodes. CD34 is normally expressed in the

umbilical cord and bone marrow on hematopoietic cells, endothelial progenitor cells, endothelial cells of blood vessels, mast cells, a subpopulation of dendritic cells in the interstitium and around the adnexa of dermis of skin as well as on cells from some soft tissue tumors (Furness et al. 2006). Therefore, it should not be expressed on MSCs.

CD45 was originally called “leukocyte common antigen” and should not be expressed on MSCs. It is expressed in various forms on all differentiated hematopoietic cells except erythrocytes and plasma cells and is believed to assist in the co-stimulation of these cells. CD45 is further expressed in lymphomas, B cell chronic lymphocytic leukemia, hairy cell leukemia and acute nonlymphocytic leukemia.

CD73 is used as a marker of lymphocyte differentiation. However, it should per definitionem be coexpressed with CD90 and CD105 at very high concentrations (> 95%) on any potential MSCs (Dominici et al. 2006).

CD90 (Thy-1) is usually used as a marker for a variety of stem cells and for the axonal processes of mature neurons. Amongst the cells expressing Thy-1 are thymocytes, prothymocytes, neurons, mesenchymal stem cells, hematopoietic stem cells, natural killer cells, murine T cells, endothelium, renal glomerular mesangial cells, circulating metastatic melanoma cells, follicular dendritic cells (FDCs), and a fraction of fibroblasts and myofibroblasts. Thy-1 can be considered one of the major markers for mesenchymal stem cells.

CD105 (endoglin) is a part of the TGF- β receptor complex. It has been found on endothelial cells, activated macrophages, fibroblasts, smooth muscle cells and mesenchymal stem cells. As endoglin has been found to be part of the TGF- β 1 receptor complex, it may be involved in the binding of TGF- β 1, TGF- β 3, BMP-2, and/or BMP-7.

CD146, also known as the melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is a cell adhesion molecule currently used as a marker for endothelial cell lineage. It is expressed on activated human T cells, endothelial progenitors such as angioblasts and mesenchymal stem cells and is further strongly expressed on blood vessel endothelium and smooth muscle. CD146 has been also demonstrated to appear on a small subset of T and B lymphocytes in the peripheral blood of healthy individuals. CD146 is considered a marker for mesenchymal stem cells isolated from multiple adult and fetal organs and its expression may be linked to multipotency; mesenchymal stem cells with greater differentiation potential express a higher level of CD146 on the cell surface (Russel et al. 2010).

STRO-1 is an antigen identified by Simmons and Torok-Storb (1991) used to label bone marrow stromal cell precursors and bone marrow stem cells (Simmons et al. 1994; Gronthos and Simmons 1996). STRO-1 is a cell surface protein expressed by bone marrow stromal cells and erythroid precursors. The frequency of colony forming units-fibroblast (CFU-F) is enriched 100-fold in the STRO-1+/Glycophorin A-population from bone marrow cells. The subset of marrow cells that expresses the STRO-1 antigen is usually capable of differentiating into multiple mesenchymal lineages including hematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts, and chondrocytes.

Taken together, the “agreed upon” combination of a negative expression of CD14, CD34, and CD45 and positive expression of CD73, CD90, CD105, CD146, and STRO-1 as identification criteria for PDSCs can be justified. The importance of CD146 and STRO-1 expression as markers for multipotency is, however, a major criterion for the identified cells. This STRO-1 positive fraction was therefore of utmost importance and the magnetic cell sorting using anti-STRO-1 antibodies in combination with anti-IgM MicroBeads was crucial to enrich it.

4.1.5 Colony-forming unit (CFU) assay

Mesenchymal stem cells first identified in aspirates of adult bone marrow were characterized, apart from their potential to undergo extensive proliferation *in vitro* and to differentiate into different stromal cell lineages, by their capacity to form clonogenic clusters of adherent fibroblastic-like cells or fibroblastic colony-forming units (CFUs) (Castro-Malaspina et al. 1980, Gronthos et al. 1995, Chen et al. 1998, Gronthos et al. 1999, Marcacci et al. 1999, Mackenzie et al. 2001, Dennis et al. 2002, Cowan et al. 2004). This is an important criterion for differentiating stem cells from the morphologically similar fibroblasts, which usually do not possess this colony forming ability, but rather grows in a scattered manner when cultured under the same culture conditions. Each colony arises from a single mesenchymal stem cell, thereby clearly demonstrating the ability of these cells to self-replicate and to generate clonogenic daughter cells.

This criterion was again taken as a marker of identification for PDSCs isolated from alveolar bone and gingival soft tissues. If the isolated cells would possess stem cell character they should firstly be able to rapidly replicate, giving rise to multiple clonogenic daughter cells. This, in turn, should subsequently be visible *in vitro* by their colony-forming ability. These clonogenic daughter cells should exhibit the ability for self-renewal through mitotic cell division and, under suitable conditions, should reveal an inherent capability to subsequently

differentiate into a diverse range of specialized cell types (Wagers et al. 2004, Cai et al. 2004, Robey et al. 2006, Morsczech et al. 2008).

4.1.6 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a molecular biological technique to amplify a single or few copies of DNA, generating thousands to millions of copies of a particular DNA sequence. This technique can detect even minute amounts of host DNA in tissue samples. The method usually relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase enable a selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

In this study LightCycler PCR (Roche Molecular Biochemicals) was used to test the gene expression profile of the isolated cells from both the alveolar bone as well as from the gingival margin for the genes of the osteogenic markers ALP, type I, III and V collagens, osteonectin, osteopontin, and osteocalcin. LightCycler conducts a real-time PCR. The procedure follows the general principle of PCR; its key feature is that the amplified DNA is detected as the reaction progresses in real-time, compared to standard PCR, where the product of the reaction is detected at its end. LightCycler amplified and monitored the development of the target nucleic acid by a fluorescence assay after each cycle (denaturation, annealing, and extension). It subsequently provided relatively rapid (30 to 40 min) results and automation of PCR by precise air-controlled temperature cycling and continuous monitoring by the fluorometer incorporated into the LightCycler. The technique is sensitive, rapid, and easy to use.

4.1.7 Multilineage differentiation potential

A major distinctive criterion for identifying stem cells is their multipotency, which is their ability to express multilineage potential and subsequently to give rise to many different tissues, while overcoming lineage boundaries. To demonstrate this ability, standard culture protocols utilizing inductive media for cell differentiation into osteoblastic, adipogenic as well as chondrogenic directions were used. The composition of the cell inductive differentiation media was deduced from standard literature and the differential staining proposed by the International Society for Cellular Therapy (Dominici et al. 2006) set as proof

for the successful differentiation. Alizarin red stain for demonstration of the osteogenic differentiation through staining calcified deposits, Oil Red O stain for adipogenic differentiation through staining lipid inclusion bodies and Alcian Blue stain for chondrogenic differentiation through staining glycosaminoglycans were performed. When feasible this study also, beside the staining procedures, conducted PCR examination to additionally affirm the differentiation potential of the isolated cells.

4.2 Results

In the present study multipotent postnatal PDSCs isolated from both the alveolar bone and the gingival cervical margin confirmed to both the criteria set by the International Society for Cellular Therapy (Dominici et al. 2006) as well as the ones defined by Bratold et al. (2006) in their review articles.

- PDSCs isolated from both sources were plastic adherent when maintained under standard culture conditions. This plastic adherence is a primary characteristic of any potential mesenchymal stem cell.
- In contrast to periodontal fibroblasts both PDSCs sources showed colony forming unit abilities similar to BMSSCs. This colony formation clearly points at the ability of these cells to divide, resulting in multiple clonogenic identical daughter progenitors. This ability is a hallmark for any stem cell, ensuring its continuous supply of cells for any regeneration process. One daughter cell remains a stem cell while the other differentiates into a committed cellular subtype (asymmetric cell division).
- More than 95% of PDSCs from both sources expressed the surface markers CD73, CD90, and CD105, as measured by flow cytometry and these cells lacked expression (less than 2%) of the surface markers CD45, CD34, and CD14. In addition, cells from both sources were positive for the surface markers STRO-1 and CD146 pointing at their multipotency. This surface marker constellation attests to the widely approved expression profile of multipotent stromal cells and clearly demonstrates the identity of the isolated cells.
- Regarding their multilineage differentiation potential, PDSCs from both sources were able to differentiate into the osteoblastic direction as evidenced by Alizarin Red staining, the adipocytic path as apparent by Oil Red O staining and LPL expression, and a chondroblastic one as evidenced by Alcian Blue staining, when cultured under

standard *in vitro* differentiating conditions. This multipotency of the isolated stem cells is of utmost importance in the field of periodontology. If these cells are to achieve regeneration, they should be able to form different tissues and structures, including cementum, PDL and alveolar bone. It is the conclusion of this study that this ability, on the basis of this differentiation potential, can be expected from the isolated PDSCs.

- The common criteria of mineralized tissue marker expression, usually used as an identification criterion for other multipotent postnatal stem cells, as PDLSCs, ADSCs and BMSSCs, were tested in the field of PDSCs identification. Regarding their gene expression profile PDSCs should express mineralized tissue markers, as ALP, type I and III collagens, osteonectin, osteopontin and osteocalcin (Bartold et al. 2006). Yet, this creates a dilemma as earlier studies reported mineralized tissue marker expression by periodontal fibroblasts, stating that they could express almost the same marker constellation (Takano-Yamamoto et al. 1994, Bronckers et al. 1994, Lallier et al. 2005).

In the present study unstimulated PDSCs from both sources as well as periodontal fibroblasts expressed mRNA of most tested mineralized tissue markers, however in different quantities.

These markers are of high importance in any periodontal regeneration process. During the regeneration process new tissues and structures ought to be re- as well as newly formed. These structures are mainly the alveolar bone and the cementum as well as a fibrous attachment, the PDL, anchoring them together. The newly formed alveolar bone as well as the cementum requires ALP, type I and III collagens, osteonectin, osteopontin, and osteocalcin. The PDL is mainly composed of type I with a cross-linkage done by type III collagens. Type V collagen also plays a crucial role here. Apart from being located on or adjacent to basement membrane, it plays a pivotal role in the PDL development, by its control of the initiation of collagen fibril assembly (Wenstrup et al. 2004). As the tissues mineralize ALP then osteonectin and osteopontin followed finally by osteocalcin appear.

As was evident by real-time PCR, PDSCs from both sources showed a tendency for a higher expression of collagen type I, III, ALP, osteopontin and osteonectin, while periodontal fibroblasts showed a higher expression of collagen V and osteocalcin and almost no expression of ALP. These findings are consistent with earlier studies on mesenchymal stem cells and the relation of their temporal expression of mineralized

tissue markers to the cellular developmental process. In terms of matrix synthesis, immature cells ought to express collagen type I and III as well as ALP, osteopontin and osteonectin, but should not highly express proteins characteristic of bone such as osteocalcin. With the decline in expression of most of these markers, appears a high expression of osteocalcin late during any mineralized tissue development, as in the periodontal tissue, denoting a shift from an immature to a more differentiated cellular state (Yoon et al. 1987, Vanvlasselaer et al. 1994, Lian et al. 1995). In this context, this shift in mineralized tissue marker expression from the immature PDSCs to the more differentiated periodontal fibroblasts could be explained.

Whether a time dependent quantification of the shift in the mineralized marker expression profile could be employed as a designator for the developmental state of the periodontal cells, remains to be investigated.

The present study aimed at broadening the scope of adult/postnatal multipotent periodontal stem cell isolation from the oral tissues, especially the periodontium. The study succeeded in isolating and characterising adult postnatal multipotent PDSCs for the first time from the alveolar bone surrounding the root surface as well as the soft tissue at the gingival cervical margin. This study thereby adds two novel sources for the isolation and expansion of PDSCs. In the previous study by Seo et al. (2004), tackling the issue of PDLSCs isolation, only a single component of the periodontium, namely the PDL, has been addressed. The alveolar bone and cervical gingival margin, sharing their developmental origin with the PDL, however, constitute pivotal components of the periodontium, and therefore serve as a source of adult/postnatal multipotent PDSCs. Their main merit over the originally proposed technique for the isolation of PDLSCs from the PDL is that they could be more readily obtained with minimally invasive procedures and, of course, without sacrificing a highly valuable tooth to obtain its PDL as described by Seo et al. (2004).

The isolation of PDSCs from these two sources as described in the two protocols therefore poses a promising, conservative, and less invasive alternative. The periodontium of a single tooth, namely alveolar bone, gingival soft tissue, and PDL, can thereby act as a single continuous unit, giving rise to a vast number of adult/postnatal multipotent periodontal stem cells. This would add to the newly developing era of tissue regeneration approaches mediated by human adult/postnatal multipotent PDSCs, to become a very conservative and practical cellular-based treatment for periodontal as well as other diseases.

5. Summary

Periodontitis is a destructive inflammatory disorder of the periodontium branded by the destruction of periodontal tissues namely the PDL, cementum, alveolar bone, and gingiva. Once these tissues are lost, the foremost goal of periodontal therapy is to regenerate the diseased tissues if possible to their original form, architecture, and function. This a demanding task, that requires the harmonization of many actions at both cellular and molecular levels.

Successful periodontal regeneration encompasses the formation of new gingival connective tissue, the restoration of bone, and most decisively, the insertion of new connective tissue fibres into newly formed cementum on the formerly diseased root surfaces. A variety of procedures to regenerate the lost tissue have been suggested, including the utilization of the principles of guided tissue regeneration and the application of an assortment of growth factors as well as bone and enamel matrix proteins on the root surfaces. However, these strategies showed only limited regeneration of periodontal tissue and were associated with incoherent and unpredictable clinical outcomes.

Stem cells are progenitor cells characterized by their ability to self-renew and differentiate to produce specialized cells. There are two main categories of stem cells, the pluripotent embryonic stem cells and the multipotent adult ones. Recently, dental tissues such as the PDL, the dental pulp and the tooth follicle have been recognized as readily available sources of adult stem cells.

Employing the criteria defined by Bartold et al. (2006) as well as the minimal standards accepted by the International Society for Cellular Therapy (Dominici et al. 2006) to define human MSCs and bearing in mind that the PDL shares the same developmental path with the cervical gingival margin and alveolar bone, the aim of this study was to present two protocols for the isolation and characterization of populations of mesenchymal multipotent stem cells-periodontal stem cells (PDSCs) derived from the alveolar bone and the cervical gingival margin of the periodontium.

In this study the sample consisted of eleven partially impacted wisdom teeth, which were surgically extracted together with a piece of alveolar bone and cervical gingival margin attached to them. Cells isolated from the alveolar bone and the cervical gingival margin of the periodontium showed adherence to tissue culture plastics under standard in vitro culture conditions. They both exhibited the ability to form colony-forming units and were highly

positive for the surface markers CD73, CD90, CD105 (> 95%), moderately positive for the markers CD146 and STRO-1, and negative for CD14, CD34, and CD45. On the mRNA level unstimulated cells from both sources expressed the osteogenic markers alkaline phosphatase, type I and III collagen, osteonectin, osteopontin, and osteocalcin. In addition, they both expressed type V collagen. Finally, cells from both sources demonstrated in vitro multilineage differentiation potential as evidenced by histological staining and PCR assays. It is concluded from this study that adult postnatal multipotent PDSCs can be readily isolated from the alveolar bone as well as from the cervical gingival margin.

6. References

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7. Appendix

7.1 Figures

Figure 1: Embryonic stem cells _____	12
Figure 2: Mesenchymal stem cells _____	13
Figure 3: MACS Method _____	27
Figure 4: Schematic drawing of (A) 6-well culture plates for osteogenic and adipogenic differentiation and (B) 1.5 ml Eppendorf tubes for chondrogenic differentiation of the isolated cells (blue masses). _____	29
Figure 5: Microscopic analysis of cells derived from the alveolar bone after 1 week and after first cell passage _____	34
Figure 6: Microscopic analysis of cells derived from the gingival margin after 1 week and after first cell passage _____	34
Figure 7: Surface markers' expression on cells derived from the alveolar bone _____	35
Figure 8: Surface marker expression of cells derived from the gingival cervical margin _____	36
Figure 9: Colony forming unit assay in cells derived from the alveolar bone _____	37
Figure 10: Colony forming unit assay in cells derived from the gingival cervical connective tissue _____	37
Figure 11: Colony forming unit assay in periodontal fibroblasts _____	37
Figure 12: Osteogenic differentiation as revealed by Alizarin Red staining of alveolar bone derived stem cells with osteogenic inductive (left) and control (right) media _____	38
Figure 13: Osteogenic differentiation as revealed by Alizarin Red staining of gingival margin derived stem cells with osteogenic inductive (left) and control (right) media _____	38
Figure 14: Adipogenic differentiation as revealed by Oil-Red-O staining of alveolar bone derived stem cells with adipogenic inductive (left) and control (right) media _____	39
Figure 15: Adipogenic differentiation as revealed by Oil-Red-O staining of gingival margin derived stem cells with adipogenic inductive (left) and control (right) media _____	39
Figure 16: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for GAPDH and LPL expression in alveolar bone derived cells with adipogenic inductive (left) and control (right) media _____	40
Figure 17: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for GAPDH and LPL expression in gingival margin derived cells with adipogenic inductive (left) and control (right) media _____	40
Figure 18: Chondrogenic differentiation as evidenced by Alcian Blue staining of alveolar bone derived stem cells with chondrogenic inductive (left) and control (right) media _____	41
Figure 19: Chondrogenic differentiation as evidenced Alcian Blue staining of gingival margin derived stem cells with chondrogenic inductive (left) and control (right) media _____	41
Figure 20: Gene expression profile of unstimulated cells derived from the gingival margin (PDSCs-Gingiva), alveolar bone (PDSCs-Bone) as well as periodontal fibroblasts	

(Fibroblasts) for Collagen I, III and V and alkaline phosphatase as determined by real-time PCR (boxplots). _____ 42

Figure 21: Gene expression profile of unstimulated cells derived from the gingival margin (PDSCs-Gingiva), alveolar bone (PDSCs-Bone) as well as periodontal fibroblasts (Fibroblasts) for osteonectin, osteopontin and osteocalcin as determined by real-time PCR (boxplots). _____ 43

Figure 22: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for (1) GAPDH, (2) Osteocalcin, (3) Osteonectin, (4) Osteopontin, (5) Collagen I, (6) Collagen V, (7) Collagen III and (8) ALP expression in unstimulated cells derived from the alveolar bone. _____ 44

Figure 23: Control-PCR: An aliquot of the real-time PCR reaction has been loaded on an agarose gel and stained with ethidiumbromide to control for the presence of a single amplification product and its expected size for (1) GAPDH, (2) Osteocalcin, (3) Osteonectin, (4) Osteopontin, (5) Collagen I, (6) Collagen V, (7) Collagen III and (8) ALP expression in unstimulated cells derived from the gingival margin. _____ 44

7.2 Table

Table 1: Primer sequences used in the PCR _____ 32

7.3 Abbreviations

ADSCs	adipose derived stromal cells
ALP	alkaline phosphatase
BMPs	bone morphogenetic proteins
BMSSCs	bone marrow stromal stem cells
BSA	bovine serum albumin
BSP	bone sialoprotein
CFU	colony forming unit
DFSCs	dental follicle stem cells
DMP1	dentine matrix protein 1
DMSO	dimethylsulfoxide
dNCPs	dentine non-collagenous proteins
DPSCs	dental pulp stem cells
DSPP	dentine sialophosphoprotein
EDTA	ethylenediaminetetraacetic acid
EMD	enamel matrix derivatives
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTR	Guided tissue regeneration
HA/TCP	hydroxyapatite/tricalcium phosphate
HSCs	hematopoietic progenitors or stem cells
IGF-I	insulin like growth factor
LPL	lipoprotein lipase
MACS	magnetic activated cell sorting
MSCs	mesenchymal stem cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelets derived growth factor
PDLSCs	periodontal ligament stem cells
PDSCs	periodontal stem cells
PLLA	Poly-L-Lactide Acid

SHEDs

stem cells from human exfoliated deciduous
teeth

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7.5 Publications, Presentations and Patents derived from this thesis:

7.5.1 Peer review articles:

- **Programmable cells of monocytic origin (PCMOs) show similarities in common surface marker expression to Periodontal Ligament Stem Cells (PDLSCs)**
Fawzy El-Sayed KM, Vöhrs H, Paris S, Becker ST, Wiltfang J, Fändrich F, Dörfer CE, Ungefroren H.
Journal of Clinical Periodontology (under Review).

7.5.2 Citable abstracts:

- **Programmable Cells of Monocytic Origin resemble MSCs rather than HSCs.**
Vöhrs H, **F. El-Sayed KM**, Ungefroren H, Möller B, Wiltfang J, Dörfer CE and Fändrich F.
Journal of Dental Research 89 (Spec Iss B):271, 2010.
- **Isolation and Expansion of Periodontal Stem Cells: Three New Techniques.**
F. El-Sayed KM, Vöhrs H, Paymard-Stolz M, Liu Q, Dörfer CE.
Journal of Dental Research 89 (Spec Iss B):3604, 2010.
- **Effect of BMP-2 and Emdogain on gene expression of PDSCs.**
Fawzy El-Sayed KM, Vöhrs H, Paymard-Stolz M, Dörfer CE and Paris S.
Journal of Dental Research 90 (Spec Iss A):14667, 2011.

7.5.3 Presentations:

- **“Isolation and Expansion of Periodontal Stem Cells: Three New Techniques”.**
International Association for Dental Research (IADR). Barcelona, Spain-July 2010.
- **“Verfahren für die Isolation und Charakterisierung multipotenter postnataler Stammzellen aus dem alveolären Knochen“**
Arbeitsgemeinschaft für Grundlagenforschung (AfG). Mainz, Germany-January 2011.
- **“Effect of BMP-2 and Emdogain on gene expression of PDSCs”.**
International Association for Dental Research (IADR). San Diego, CA, USA-March 2011.

7.5.4. Patents:

- Provisional patent entitled: **“New Techniques for the Isolation of Periodontal Stem Cells”.**

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7.7 List of publications

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Aboulela S, Elbeialy AR, **F. El-Sayed KM**, Selim EN, Elmangoury NH, Mostafa YA. American Journal of Orthodontics and Dentofacial Orthopaedics (in press)

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Graetz G, Dörfer CE, Kocher T, **Fawzy El-Sayed K**, Wiebe JF, Gomer K, Rühling A. Journal of Clinical Periodontology (in press).

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Citable abstracts:

Programmable Cells of Monocytic Origin resemble MSCs rather than HSCs.

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Journal of Dental Research 90 (Spec Iss A):147903, 2011.